Farnesoid X Receptor activation protects against intestinal inflammation: potential mechanisms and therapeutic implications

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Financial support by the Department of Metabolic and Endocrine Diseases (UMC Utrecht), the Department of Gastroenterology and Hepatology (UMC Utrecht), Abbott, Zambon, Bayer, Tramedico for publication of this thesis is gratefully acknowledged.

Cover: "DNAbsolute"
by Raffaella Gadaleta
ISBN: 978-90-8891-272-6
Printed by: Proefschriftmaken.nl || Printyourthesis.com
Published by: Uitgeverij BOXPress, Oisterwijk

Farnesoid X Receptor activation protects against intestinal inflammation: potential mechanisms and therapeutic implications

Farnesoid X Receptor activatie beschermt tegen darm inflammatie: mogelijke mechanismen en therapeutische implicaties
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 1 Juni 2011 des middags te 2.30 uur

door

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geboren op 2 Februari 1980, te Bari (Italië)

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	Prof. Dr. R. Berger
Co-promotoren:	Dr. S.W.C. van Mil
	Dr. K.J. van Erpecum

“Think of your origins: you’re not just anyone.
You weren’t born to live like brutes;
to pursue knowledge and virtue is your mission.”

“Considerate la vostra semenza:
fatti non foste a viver come bruti
ma per seguir virtute e canoscenza.”

Dante Alighieri, Inferno XXVI

A mia sorella Valentina...

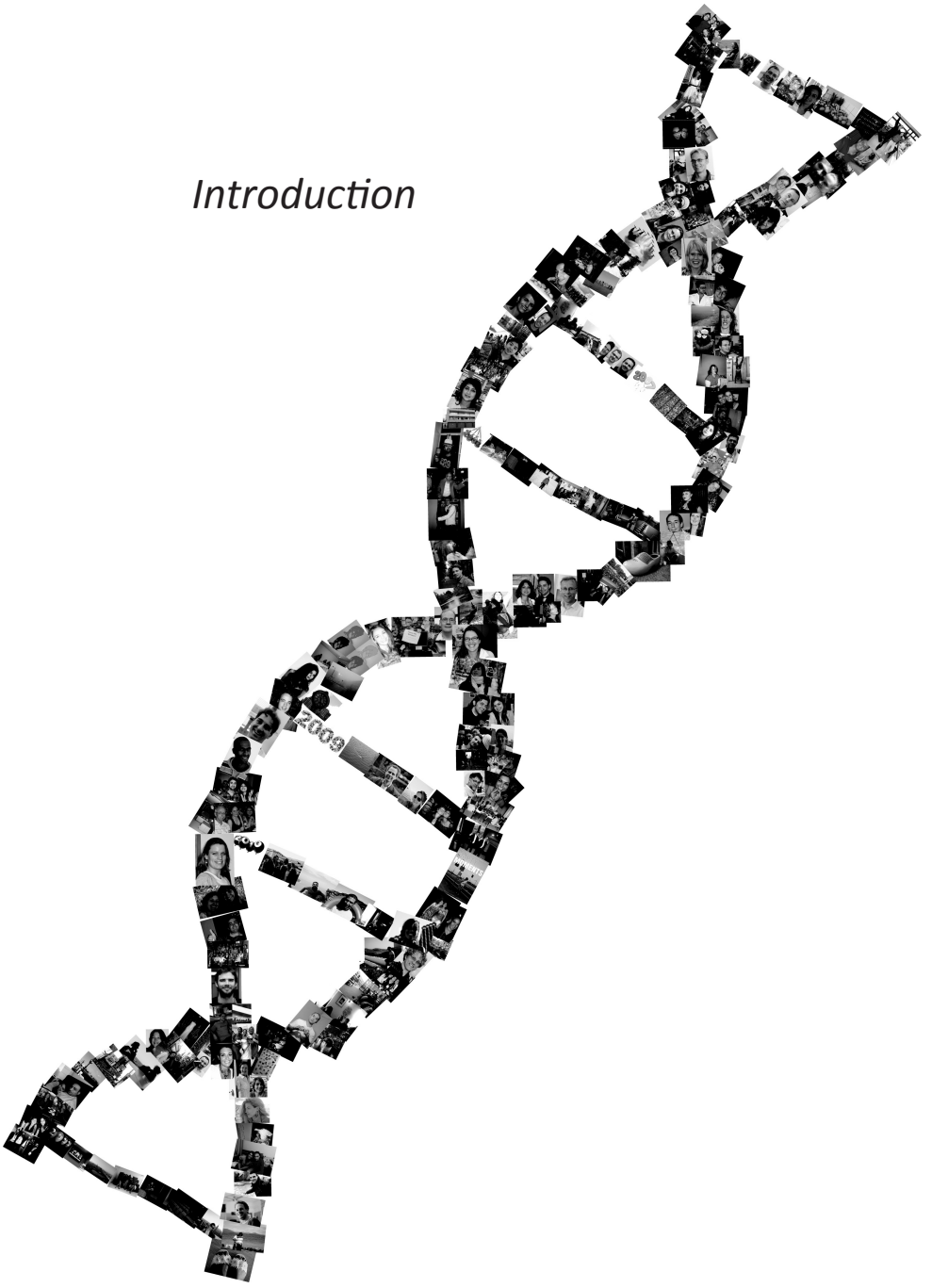
Table of contents

List of abbreviations

6-ECDCA:	6-ethyl chenodeoxycholic acid
ABC:	ATP binding cassette
ANG1:	Angiogenin 1
ASBT:	Apical Sodium-dependent Bile acid Transporter
ATP8B1:	Adenosine Tri-Phosphatase 8B1
BA:	Bile acid
BACS:	Bile Acid CoA Synthase
BAAT:	Bile Acid CoA Aminoacid N-Acyl Transferase
BDL:	Bile duct ligation
CA:	Cholic acid
CAR12:	Carbonic anhydrase 12
CCDN1:	Cyclin D1
CD:	Crohn's disease
CDCA:	Chenodeoxycholic acid
CDX:	Caudal related homeobox
CH:	Cholesterol
Co-A:	Co-activator
CoR:	Co-repressor
COX2:	Cyclooxygenase 2
Cyp3A4:	Cytochrome P450 3A4
CYP7A1:	Cholesterol 7α-Hydroxylase
CYP8B:	Sterol 12α-Hydroxylase
DBD:	DNA Binding Domain
DMSO:	Dimethyl-sulfoxide
DSS:	Dextran sodium sulphate
EV:	Empty vector
F-1-6 biPase:	Fructose 1-6 bi-Phosphate
FGF:	Fibroblast Growth Factor
FGFR:	Fibroblast Growth Factor Receptor
FITC-dextran:	Fluorescein isothyiocyanate-conjugated dextran
FXR:	Farnesoid X Receptor
FXRE:	Farnesoid X receptor responsive element
G6Pase:	Glucose 6 Phosphatase
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
GST:	Glutathione S-transferase
HPLC:	High performance liquid chromatography
HPRT:	Hypoxanthine-guanine phosphorybosil-transferase
IBABP:	Ileal Bile Acid Binding Protein
IBD:	Inflammatory bowel disease
IFNγ:	Interferon gamma

IL:	Interleukin
ILBP:	Ileal Lipid Binding Protein
iNOs:	Inducible Nitric Oxide synthase
IP-10:	Interferon-inducible Protein 10
IR:	Inverted Repeat
ko:	Knock out
LBD:	Ligand Binding Domain
LDL:	low density lipoprotein
LDH:	lactate dehydrogenase
LPMC:	lamina propria mononuclear cell
LPS:	Lipopolysaccharide
LXR:	Liver X Receptor
MCP-1:	Monocyte attractant protein-1
MPO:	Myeloperoxidase
NAFL:	Non-alcoholic fatty liver
NASH:	Non-alcoholic steato-hepatitis
NFκB:	Nuclear Factor kappa B
NOD2:	Nucleotide Oligomerization Domain2
NR:	Nuclear Receptor
NTCP:	Sodium Taurocholate Cotransporter Polypeptide
OATP:	Organic Anion Transporting Polypeptide
OST:	Organic Solute Transporter
PAMP:	Pathogen associated molecular pattern
PBMC:	Peripheral blood mononuclear cell
PC:	Phosphatidylcholine
PEPCK:	Phosphoenol-Pyruvate Carboxy-Kinase
PFIC:	Progressive familial intrahepatic colestasis
PLTP:	Phospholipid transfer protein
PKCζ:	Protein kinase C Zeta
PPAR:	Peroxisome Proliferator-Activated Receptor
PS:	Phosphatidylserine
RE:	Responsive Element
RXR:	Retinoid X Receptor
SHP:	Small Heterodimer Partner
SI:	Sucrose isomaltase
SREBP:	Sterol Regulatory Element Binding Protein
SULT2A1:	Sulfo-Transferase 2A1
SXR:	Steroid and Xenobiotic Receptor
TNBS:	Trinitrobenzene sulphonic acid
TNFα:	Tumor necrosis factor alpha
UC:	Ulcerative colitis
UGT2B4:	UDP Glucurunosyl-Transferase 2 B4
VPAC1:	Vasoactive intestinal peptide receptor 1
WT:	Wild type

Introduction



INTRODUCTION

The gastrointestinal tract hosts up to 1012 microbial organisms packed together per gram of luminal content[1], constituting the intestinal ecosystem, which normally establishes a symbiotic beneficial relationship with the host. The intestinal epithelial barrier at the interface between the intestinal lumen and the lymphoid tissue associated with the gastrointestinal system plays a crucial role in protecting the host from harmful pathogens while maintaining tolerance to harmless organisms[2]. In addition, mucin-secreting goblet cells generate the mucus layer that protects the mucosal surface from antigens and pathogens, while Paneth cells secrete antimicrobial peptides thereby contributing in maintaining the intestinal barrier function[3,4]. The intestinal lamina propria lies beneath the intestinal epithelium, contains several populations of immune cells and keeps the intestine in a state of controlled inflammation. Chronic and acute inflammatory intestinal disorders, such as inflammatory bowel disease (IBD), may be associated with impairment of this delicate regulation[5]. IBD is a chronic intestinal disorder with a prevalence up to 395/100 million people worldwide[6]. It is characterized by periods of activation and remission of intestinal inflammation, with potentially severe complications and even mortality[7]. It is thought to result from a combination of mucosal immune system dysregulation and compromised intestinal epithelial barrier function in genetically predisposed individuals[5]. IBD consists of two major distinct phenotypes, Crohn's disease (CD) and ulcerative colitis (UC). Inflammation in CD can potentially involve any part of the gastrointestinal tract, but it frequently affects the distal ileum and/or parts or the colon. The inflammation is often transmural and associated with strictures and fistulas. UC is generally limited to the colon or rectum and the inflammation is typically located in the mucosa[5]. In IBD, disregulated activation of the nuclear transcription factor kappa B (NF-κB) has been identified as one of the key factors in the pro-inflammatory response, resulting in strongly enhanced expression of pro-inflammatory genes, and recruitment of excess inflammatory cells to the intestinal wall[8]. Bile salts are synthesized from cholesterol in the liver, stored in the gallbladder, and released into the intestine after food intake in order to facilitate absorption of dietary lipids and liposoluble vitamins. Bile salts have a crucial function in humans, however they are also cytotoxic and their levels are therefore tightly regulated. Key in the regulation of bile salt

homeostasis is a family member of the nuclear receptor superfamily, the Farnesoid X receptor (FXR)[9-11]. Nuclear receptors (NRs) are ligand-activated transcription factors, which have vital roles in growth, development and homeostasis[12,13]. The NR superfamily has a high potential for drug targeting, because their lipophillic ligands can easily pass biological membranes. Currently, a striking 13% of all FDA approved drugs target NRs[14]. The bile salt nuclear Farnesoid X Receptor (FXR) is the master regulator of bile salt homeostasis and is mainly expressed in the ileum and liver. Once activated by bile salts, it regulates transcription of genes involved in bile salt synthesis, transport and metabolism[15]. In addition, FXR regulates lipid and glucose homeostasis, by regulating transcription of genes such as SREBP-1c and G6Pase[16,17]. Transcriptional activation of FXR involves the recruitment and binding of co-activators and release of co-repressors from target gene promoters[18-22]. Nuclear receptor activation is also regulated by post-translational modifications (PTMs), such as phosphorylation, acetylation or sumoylation. Recently, it has been established that FXR can be phosphorylated, acetylated and sumoylated.[23-25] Nevertheless, these molecular pathways are largely elusive. In addition to their role in digestion of food, bile salts together with gastric acid, pancreatic secretion, intestinal motility and local immunity are known to maintain the physiological balance of the gut microflora and the integrity of the intestinal epithelial barrier. It is well known that intestinal bile salt deficiency is associated with bacterial overgrowth and compromised barrier function, which can be reversed by oral bile salt feeding[26-29]. Interestingly, functional cross-talk between NF-κB and several nuclear receptors has been demonstrated and were shown to have anti-inflammatory properties[30,31].

The aim of this thesis is to study the role of FXR in maintaining a healthy gut and the potential therapeutic implications of FXR pharmacological activation for inflammatory bowel diseases.

In **Chapter 1**, we review the current status of the literature on the relevance of FXR activity in hepatobiliary and intestinal disease. In the subsequent chapters, we address the following research questions:

Does FXR activation ameliorate intestinal inflammation? Does FXR activation suppress the NF-κB inflammatory pathway?
These questions are addressed in **Chapter 2**, where we investigated the

role of pharmacological FXR activation in counteracting the inflammatory response in two models of murine colitis.

Is FXR activation impaired under intestinal inflammatory conditions?
In **Chapter 3**, we examined whether there is mutual antagonism between NF-κB and FXR, as has been reported for other nuclear receptors. By complementary *in vitro*, *ex vivo* and *in vivo* approaches, we investigated whether ileal FXR expression or activation is impaired under inflammatory conditions.

Is FXR activation repressed in IBD patients and are FXR polymorphisms associated with IBD?
In **Chapter 4**, we analyzed *FXR* and FXR target gene *SHP* expression in ileal and colonic biopsies of IBD patients. We hypothesized that genetic variation in FXR may confer susceptibility to IBD and we therefore evaluated whether polymorphisms in the FXR gene are associated with specific phenotypic subgroups of Crohn's disease and ulcerative colitis.

Is FXR expression and/or activation impaired in IBD patients with concurrent liver disease?
A significant proportion of IBD patients are suffering from primary sclerosing cholangitis (PSC) as well. We hypothesized that PSC patients with IBD may also have decreased FXR activation in the intestine due to a relative excess of primary bile salts and decreased bile salt excretion in the intestine. In addition, we studied the effect of treatment with ursodeoxycholic acid (UDCA) in these patients, which is known to be a poor ligand for FXR, since it might also lead to a decreased FXR activity in the intestine . In **Chapter 5**, we studied intestinal FXR expression and/or activation in PSC patients treated with UDCA. In addition, we studied in PSC patients the effect of UDCA treatment on biliary bile salt and phosphatidylcholine molecular species composition.

What are the molecular mechanisms underlying FXR transactivation and transrepression?
Besides classical FXR transactivation of target genes, in Chapter 2 we described a novel mechanism by which FXR regulates transcription, by transrepression of

NF-κB signalling. In **Chapter 6**, we start dissecting the differential mechanisms for classical transactivation and transrepression of FXR, by identification and characterization of post-translational modifications (PTMs) in FXR.

Is FXR a valuable target for the treatment of intestinal inflammation?
In the summarizing discussion (**Chapter 7**) the classical role of FXR controlling bile salt homeostasis and the new role of FXR in intestinal inflammation are discussed. Based on literature and the results presented in this thesis, new therapeutical strategies for the treatment of intestinal inflammation are proposed.

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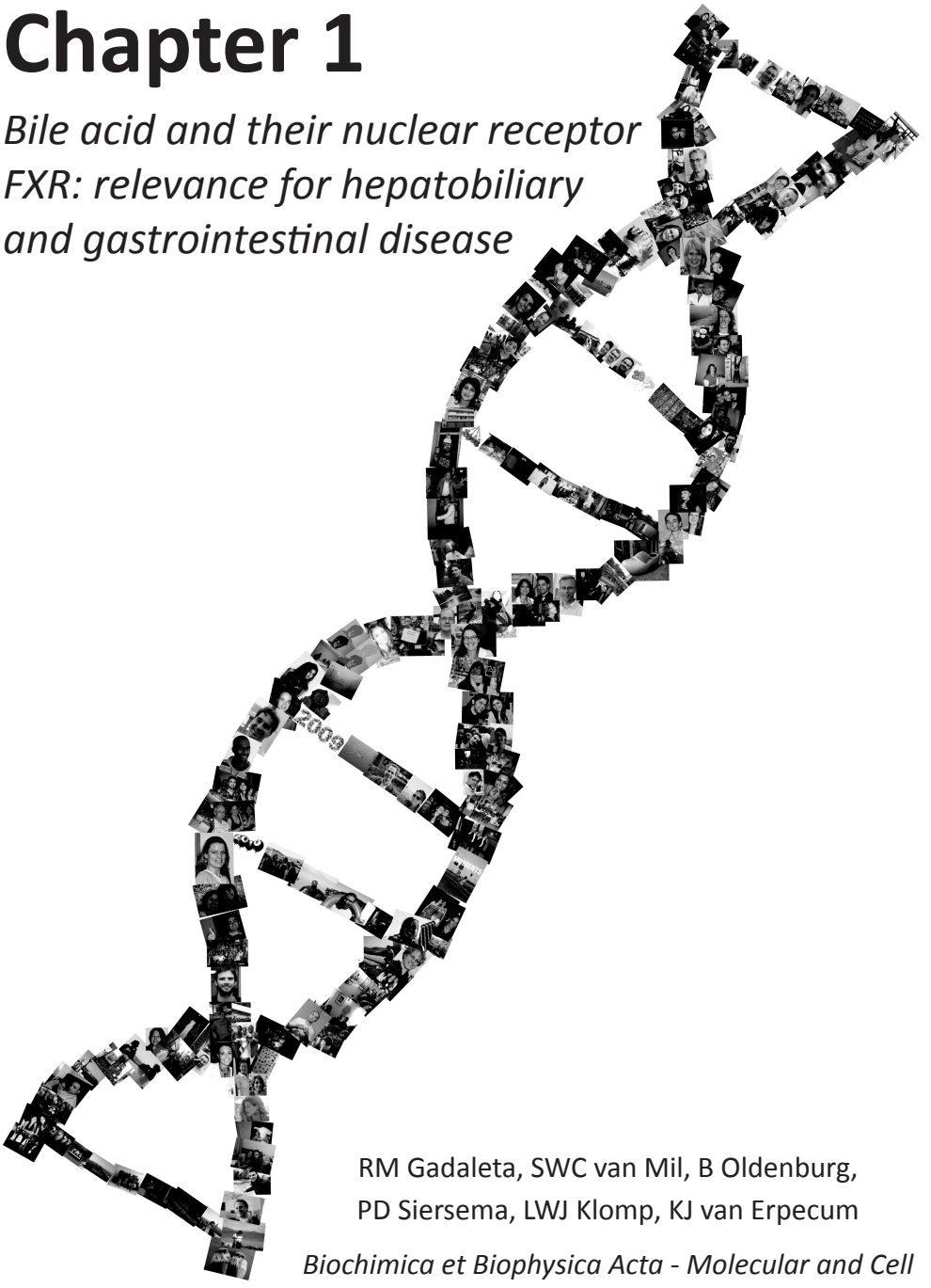
Chapter 1

Bile acid and their nuclear receptor FXR: relevance for hepatobiliary and gastrointestinal disease



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Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. July 2010

ABSTRACT

The nuclear receptor Farnesoid X Receptor (FXR) critically regulates nascent bile formation and bile acid enterohepatic circulation. Bile acids and FXR play a pivotal role in regulating hepatic inflammation and regeneration as well as in regulating extent of inflammatory responses, barrier function and prevention of bacterial translocation in the intestinal tract. Recent evidence suggests that the bile acid – FXR interaction is involved in the pathophysiology of a wide range of diseases of the liver, biliary and gastrointestinal tract, such as cholestatic and inflammatory liver diseases and hepatocellular carcinoma, inflammatory bowel disease and inflammation-associated cancer of the colon and esophagus. In this review we discuss current knowledge of the role the bile acid–FXR interaction in patho-physiology of the liver, biliary and gastrointestinal tract, and proposed underlying mechanisms, based on *in vitro* data and experimental animal models. Given the availability of highly potent synthetic FXR agonists, we focus particularly on potential relevance for human disease.

1. Introduction

Bile acids are involved in nascent bile formation, biliary cholesterol solubilisation and intestinal absorption of lipids and lipid-soluble molecules. Various transport proteins for bile acids and the other major bile lipids (phosphatidylcholine and cholesterol) have been identified in the liver, which are tightly regulated by nuclear receptors such as the bile acid nuclear receptor Farnesoid X Receptor (FXR) and the Liver X Receptor (LXR). Bile acids may exhibit devastating effects, especially in organs exposed to high flux of these detergent molecules. Indeed, disturbances in biliary bile acid or phosphatidylcholine secretion may lead to decreased bile flow ("cholestasis") and liver disease. Currently, bile acids are also increasingly recognized as signalling molecules in a wide range of fields, such as energy homeostasis and metabolism of glucose and lipids, and bile acid-mediated activation of FXR is a major underlying pathway for these effects[1]. FXR has also been suggested to counteract pro-inflammatory and pro-atherogenic responses in cardiovascular disease[2]. Moreover, the bile acid–FXR interaction plays a pivotal role in regulating liver inflammation and regeneration, as well as in regulating the extent of inflammatory responses, barrier function and prevention of bacterial translocation in the intestinal tract. In the current review, we will focus on bile acids, FXR and the enterohepatic circulation, with special emphasis on their role in pathophysiologic derangements of the liver and the gastrointestinal tract.

2. Enterohepatic circulation of bile acids

2.1 Nascent bile formation.

Primary bile acids (in humans chenodeoxycholate and cholate) are the end products of cholesterol catabolism, which are conjugated in the liver to taurine or glycine to increase their water solubility. As shown in Figure 1, nascent bile formation occurs at the level of the hepatocytic canalicular (apical) membrane, driven by active secretion of bile acids by the energy-dependent ATP Binding Cassette (ABC) transporter ABCB11[3]. The other two major lipids secreted into human bile are cholesterol (by canalicular membrane transporter ABCG5/ABCG8[4]) and phosphatidylcholine (by canalicular membrane transporter ABCB4 which functions as a floppase, transferring this phospholipid from the inner to the outer leaflet of the canalicular membrane[5].

Since cholesterol is virtually insoluble in an aqueous environment, it is incorporated in bile in mixed micelles with bile acids and phosphatidylcholine and/or in phosphatidylcholine vesicles. In case of excess cholesterol secretion or insufficient solubilizing bile acid and/or phosphatidylcholine secretion, biliary cholesterol supersaturation occurs. Cholesterol crystals may then nucleate from aggregated vesicles which have become instable because they contain excess cholesterol (vesicular cholesterol/phosphatidylcholine ratio >1). This is the first step towards cholesterol gallstone formation. Apart from its role in cholesterol solubilisation, biliary phosphatidylcholine also protects against against bile acid cytotoxicity: whereas "simple" bile acid micelles and bile acid monomers (so called "intermixed micellar-vesicular fraction") are cytotoxic, this is not the case for mixed bile acid-phosphatidylcholine micelles[6]. Of note, the hepatocytic canalicular membrane also contains the phospholipid-flippase ATP8B1, thought to specifically translocate phosphatidylserine from the outer to the inner leaflet of the plasma membranes, with the result that the outer leaflet is enriched in phosphatidylcholine, sphingomyelin and cholesterol[7]. Cholesterol has a high affinity for sphingomyelin, and both are thought to be preferentially located in laterally separated microdomains[8,9]. These detergent-resistant microdomains offer additional protection against bile acids and are essential for normal function of transmembrane transporters[10]. Recent evidence indicates that hepatocytic canalicular ABC-transporters are localized within these microdomains[11]. For further details on bile formation and biliary cholesterol solubilisation we refer to reviews[12,13].

2.2 Enterohepatic circulation.

Nascent canalicular bile is subsequently modified in the biliary tract. In the fasting state, human bile is temporary stored in the gallbladder, with 3-10 fold concentration due to absorption of water. Upon meal ingestion, the hormone cholecystokinin is released from the proximal intestinal tract, with gallbladder contraction and delivery of bile to the intestine and transition from the fasting to the fed state. In the intestinal lumen, bile acids play an essential role for solubilisation and absorption of lipids and lipid-soluble vitamins. As shown in Figure 1, bile acids are conserved in an efficient enterohepatic circulation: in the ileum they are reabsorbed by the Apical Sodium-dependent Bile Acid Transporter (ASBT)[14]. In the enterocyte, bile acids activate the bile acid nuclear receptor Farnesoid X Receptor (FXR, see Section 2.3). FXR then

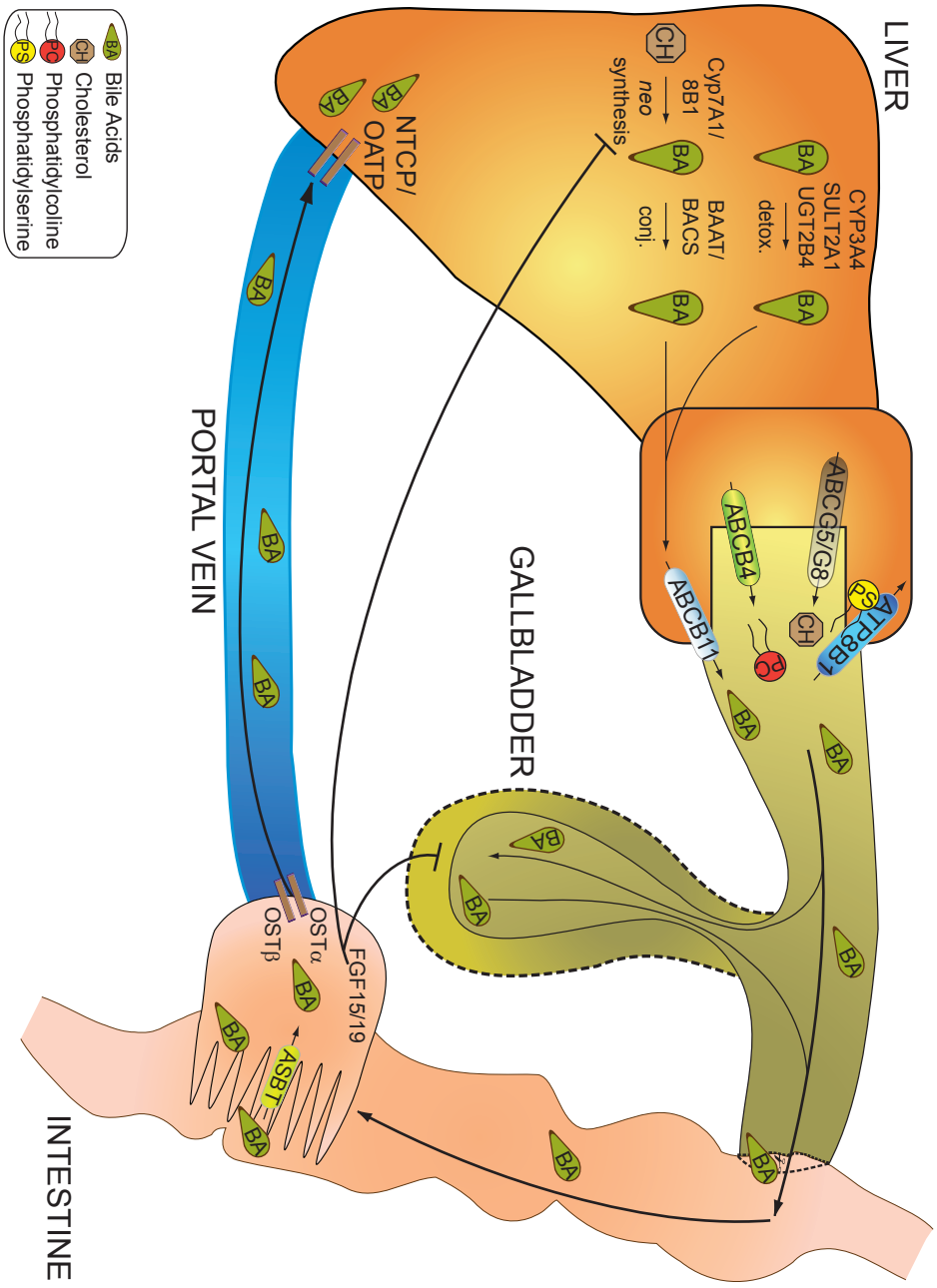

Figure 1: *Nascent bile formation and enterohepatic circulation.*
At the hepatocytic canalicular membrane, phospholipid asymmetry is maintained by flippase ATP8B1, translocating phosphatidylserine from the outer to the inner leaflet. Bile

acids, phosphatidylcholine and cholesterol are secreted into bile by transport proteins ABCB11, ABCB4 and ABCG5-G8. In the fasting state, bile acids are temporally stored in the gallbladder. Upon feeding, gallbladder contraction occurs, with release of bile acids into the intestine. In the ileum, bile acids are conserved in the enterohepatic circulation by transport proteins ASBT and OSTα-OSTβ at the apical resp. basolateral membranes of the enterocyte, transported via the portal vein to the liver and reabsorbed by transport protein NTCP (for details see Sections 2.1 and 2.2). In the liver, neosynthesis, conjugation and detoxification of bile acids occur. In the enterocyte, bile acid-dependent FXR activation (see Section 2.3 and Figure 2) results in production/secretion of FGF15/19. This hormone exerts negative feedback on hepatic bile salt neosynthesis and induces gallbladder dilatation, signalling end of the fed state and transition to the fasting state.

induces the expression of Fibroblast Growth Factor (FGF)15/19 (mouse and human orthologous, respectively). This hormone exerts negative feed back on bile acid neosynthesis in the liver after binding to FGF receptor 4 (see Section 2.3), induces gallbladder dilatation after binding to FGF receptor 3[15] and may also inhibit pancreatic stimulation. Infact, FGF15/19 may function as an "ileal brake", by signalling progression from the fed ("digestive") to the fasting ("interdigestive") state, including appropriate changes of the bile acid enterohepatic circulation. After their reabsorption, bile acids are secreted from the enterocyte into portal blood by the basolateral heterodimeric Organic Solute Transporters OSTα-OSTβ[16] and transported back to the liver, where the great majority is reabsorbed by basolateral transporters such as the sodium (Na)-Taurocholate Cotransporter Protein (NTCP).

2.3 The nuclear receptor FXR.

The bile acid enterohepatic circulation and the process of bile formation are tightly regulated, depending on modulating dietary and hormonal signals. Nuclear receptors are the key regulators in this respect: when activated by specific ligands, they prime the activation of multiple genes with accurate, synchronized and coherent functional responses and close coordination between liver and intestine as a result.
The liver X Receptor (LXR) is activated by oxysterols which are synthesized in the cell under conditions of cholesterol excess. After forming a heterodimer with Retinoid X Receptor (RXR), the LXR-RXR complex promotes biliary cholesterol secretion, through the transcriptional upregulation of ABCG5/ABCG8 hepatic canalicular transporter.
The bile acid nuclear receptor Farnesoid X Receptor (FXR) is the master

regulator of bile acid homeostasis. FXR was cloned in 1998 and originally classified as an "orphan" nuclear receptor: its cognate ligand was not known, despite weak activation by an intermediate of the mevalonate pathway, farnesol[17]. Later, FXR proved to be a bile acid nuclear receptor[18,19]. There are two genes encoding for FXR: FXRα and FXRβ. The FXRβ gene is only functional in rabbits, rodents and dogs, while it is a pseudogene in humans and primates. In this review we will further refer only to the FXRα form. The FXRα gene has two functional promoters and undergoes alternative splicing, with four possible isoforms as a result[20]. FXR, as all nuclear receptors, has a well characterized structure[21]: the C-terminal ligand-binding domain (LBD) forms a hydrophobic pocket for ligand recognition and lodging. Following interaction between bile acids and the LBD, the DNA binding domain (DBD) allows gene transcription at specific sites: response elements (REs) which in the case of FXR are composed of inverted repeat elements of the canonical AGGTCA sequence, separated by one nucleotide (IR-1). The hinge region connects the DBD and the LBD. Co-regulating proteins with activation or repression function further regulate gene transcription. The ligand-independent N-terminal trans-activation domain (AF1) and the ligand-induced trans-activation domain (AF2) are the regions in the FXR molecule responsible for interactions with such regulatory proteins. The transcriptional activation of AF-1 is normally very weak, but it works synergistically with AF-2 in the LBD to more effectively upregulate gene expression. FXR needs to form an obligate heterodimer with RXR to be effective. FXR is expressed along the entire gastrointestinal tract, but at particularly high level in the liver and the ileum. Concentrations of bile acids within enterocytes and hepatocytes and within the bile acid enterohepatic circulation are tightly regulated, and FXR plays a key role in this respect (Figure 2). At the intestinal level, FXR activation increases expression of ileal bile acid binding protein (IBABP) and basolateral OSTα-OSTβ. The role FXR in negative regulation of intestinal apical and hepatic sinusoidal bile acid uptake (mediated by ASBT and NTCP respectively) is more complex, with considerable species differences[22-27]. Nevertheless, FXR likely inhibits ASBT expression in most instances. Furthermore, FXR in the enterocyte induces expression of fibroblast growth factor (FGF)15/19: this hormone reaches the liver after secretion by the enterocyte and suppresses hepatic bile acid neosynthesis after binding to FGF receptor 4 (see Section 2.2). Recent evidence from patients with extrahepatic cholestasis

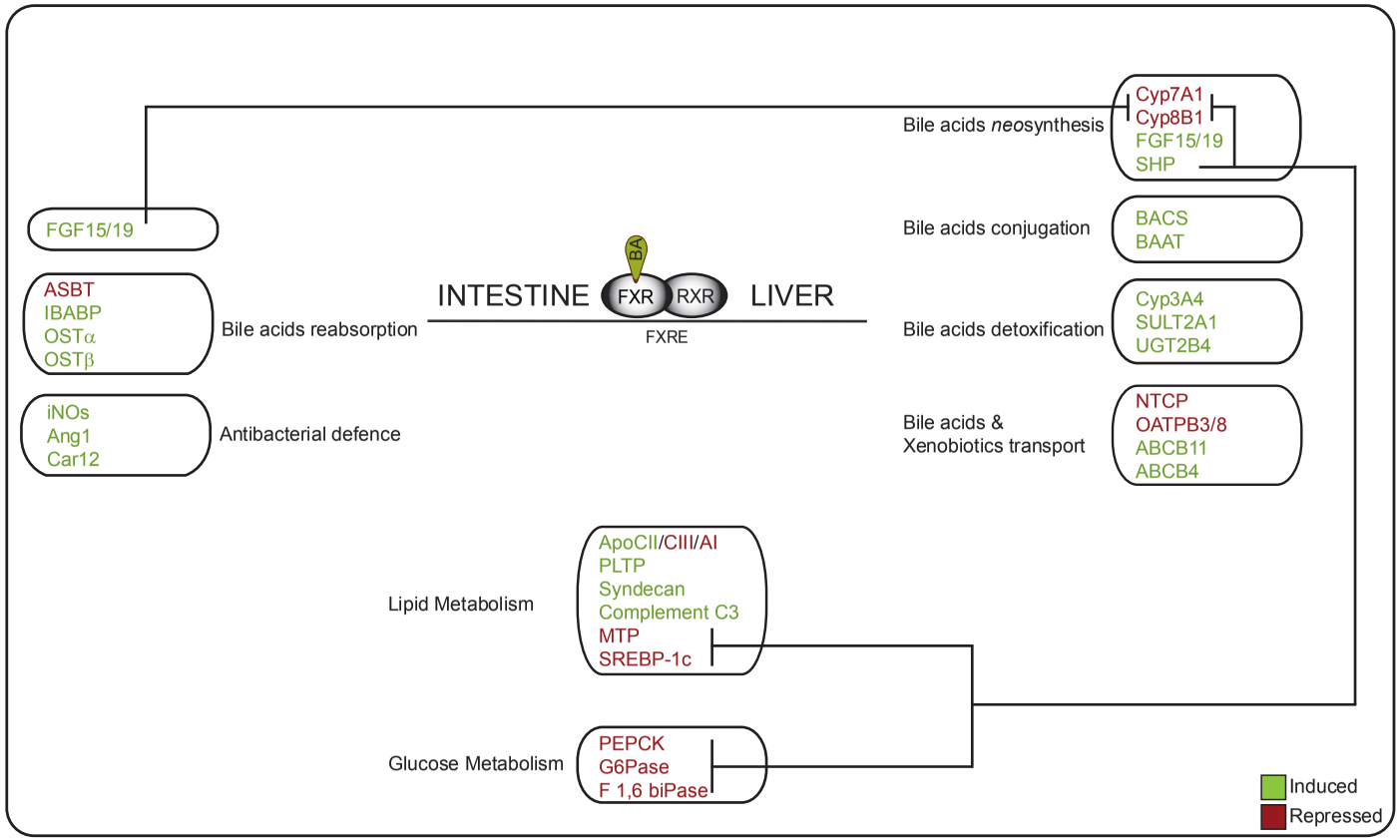
Bile acids *neo*synthesis
Cyp7A1
Cyp8B1
FGF15/19
SHP
FGF15/19
Bile acids conjugation
BACS
BAAT
ASBT
IBABP
OSTα
OSTβ
Bile acids reabsorption
INTESTINE
BA
FXR
RXR
LIVER
FXRE
Bile acids detoxification
Cyp3A4
SULT2A1
UGT2B4
iNOs
Ang1
Car12
Antibacterial defence
Bile acids &
Xenobiotics transport
NTCP
OATPB3/8
ABCB11
ABCB4
Lipid Metabolism
ApoCII/CIII/AI
PLTP
Syndecan
Complement C3
MTP
SREBP-1c
Glucose Metabolism
PEPCK
G6Pase
F 1,6 biPase
Induced
Repressed

Figure 2: *Nuclear receptor FXR binds as heterodimer with RXR on the DNA to FXR Responsive Elements (FXRE).*
Upon binding of bile acid, FXR transcriptional activity turns on, resulting in cross-talk between liver and intestine. FXR regulates bile acid enterohepatic circulation by affecting expression of various proteins in the intestine and liver involved in bile acid transport, neosynthesis, conjugation and detoxification. Also, FXR affects antibacterial defence, lipid and glucose metabolism (For details see Section 2.3).

indicates that local FXR- dependent FGF15/19 production in the liver also contributes to inhibition of bile acid neosynthesis under these pathologi cal circumstances[28]. In the hepatocyte, FXR activation inhibits, through activation of intermediate Small Heterodimer Partner (SHP), bile acid neosynthesis by inhibiting both key regulatory enzymes CYP7A1 and CYP8B1. Furthermore, FXR regulates bile acid conjugation and detoxification in the liver (see Figure 2). Last, FXR induces expression of the hepatocytic canalicular bile acid transport protein ABCB11 and phospholipid-floppase ABCB4. As a result, cytotoxicity of detergent bile acid molecules in the biliary tract is prevented. Taken together, FXR mediates intense crosstalk between the intestine and the liver, thus allowing tight regulation of bile acid enterohepatic circulation and preventing toxic effects of detergent bile acid molecules on hepatocytes and cell lining the intestinal or biliary tract.

3. Relevance of FXR for diseases of the liver and the biliary tract

3.1 Cholestasis.

Although prevalence of cholestatic liver diseases is low, they are a frequent indication for liver transplantation. The reason is the current absence of effective pharmacological options to prevent progressive disease. Cholestasis is defined as impaired bile secretion and flow. As a result of cholestasis, intrahepatic accumulation of bile acids may occur, and potentially cause fibrosis, inflammation and cirrhosis. From a teleological point of view, changes in transporter expression during cholestasis represent a compensatory -anticholestatic- response that aims to limit hepatocellular accumulation of potentially toxic biliary constituents and to provide alternative excretory routes. However, in case of cholestasis, amounts of bile acids within the intestinal lumen are often decreased, and it has been suggested that decreased bile acid absorption and insufficient

FXR-dependent FGF15/19 secretion by the enterocyte could lead to a vicious circle of inappropriately high hepatic bile acid neosynthesis and progressive liver damage. Nevertheless, it remains controversial, whether cholestasis leads to decreased or increased ileal bile acid reabsorption *in vivo*[29]. Normally, only small amounts of bile acids in the portal vein escape hepatic uptake. In contrast, serum bile acid concentrations may increase markedly in cholestatic liver disease, with deleterious effects such as (often severe) itching. Importantly, high intracellular bile acid concentrations are cytotoxic, with progressive liver disease and possibly, need for liver transplant as a result.

3.1.1 Animal models for cholestasis.

Experimental support for a role of FXR in pathogenesis of cholestasis comes from two animal models: bile duct ligation and α-naphthylisothiocyanate (ANIT) treatment[30]. In these models, the synthetic FXR agonist GW4064 markedly reduces liver injury. Both models represent an acute form of cholestasis of short duration. In contrast, 17α-ethinylestradiol-induced cholestasis is a more chronic rat model, with minimal or absent alteration of liver morphology. In the latter model, the FXR agonist 6-ethyl chenodeoxycholic acid (6-ECDCA) protects from cholestasis by increasing the expression of SHP, apical bile acid transporter ABCB11 and apical phospholipid transporter ABCB4, while reducing the expression of CYP7A1 and CYP8B (bile acid neosynthesis) or NTCP (basolateral bile acid uptake)[31].

3.1.2 Hepatocytic canalicular transport proteins and cholestasis.

Rare mutations in genes encoding various transport proteins in the hepatocytic apical membrane (see Section 2.1) may cause cholestasis. The canalicular membrane of both humans and mice with mutations in ATP8B1 resp. Atp8b1 (encoding aminophospholipid flippase in the hepatocytic canalicular membrane) exhibits decreased resistance to hydrophobic bile acids, as evidenced by enhanced biliary secretion of canalicular ectoenzymes, cholesterol and unusual phospholipids (sphingomyelin, phosphatidylserine)[32-34]. Disruption of lipid asymmetry and reduction of cholesterol content in the apical membrane by mutations in the ATP8B1 gene may apparently decrease the function of resident proteins such as the bile acid export pump, resulting in progressive familial intrahepatic cholestasis (PFIC1)[35,36]. Alternatively, it has been proposed, based on analysis of a limited

number of ileal tissue samples of PFIC1 patients and complementary *in vitro* studies, that ATP8B1 function may normally activate PKCζ with subsequent phosphorylation, nuclear localization and activation of FXR, and that this cascade is deficient in PFIC1 patients. The resulting decreased expression of downstream targets of FXR, such as ASBT and ABCB11, could underlie the cholestasis in these patients[37-39]. Indeed, transfection of HepG2 cells with ATP8B1 siRNA markedly reduces mRNA and protein expression of ABCB11, concomitant with reduced FXR expression[40]. Nevertheless, relevance of these findings for the PFIC1 phenotype is controversial[41]. Mutations in ABCB11 (encoding apical bile acid transporter) or ABCB4 (encoding apical phosphatidylcholine transporter) lead to progressive familial intrahepatic cholestasis (PFIC) type 2 or 3 respectively[42]. Since contents in bile of cholesterol-solubilizing bile acids (in PFIC2) or phosphatidylcholine (in PFIC3) are decreased, cholesterol supersaturation and cholesterol gallstones are frequently found under these circumstances. Exogenous causes (e.g. medication) may also lead to cholestasis in susceptible patients. Interestingly, recent evidence indicates frequent mutations in ABCB11 (bile acid export pump) and ABCB4 (phosphatidylcholine floppase) in patients with drug-induced cholestasis, suggesting that these transporters play a pathophysiological role in the development of drug-induced liver injury[43].

3.1.3 Intrahepatic cholestasis of pregnancy.

This pathological condition occurs in approximately one of each 200 pregnancies in Caucasians. Patients exhibit -sometimes severe- itching, with elevated maternal and fetal bile acids. van Mil *et al.* recently described four new heterozygous functional variants of FXR in patients with this condition[44]. Furthermore, their characterization of these variants revealed functional defects in either translational efficiency or activity for three of the four variants (-1>g, M1V, M173T).

3.1.4 Primary cholestatic diseases.

The primary cholestatic diseases primary biliary cirrhosis and primary sclerosing cholangitis are characterized by inflammation in the vicinity of the bile ducts, with increased expression of pro-inflammatory cytokines like TNFα or IL-1β, and accumulation of gut-derived pathogen associated molecular patterns (PAMPs) in the biliary tree. Normally, PAMPs and bacteria

are cleared from the portal blood by Kupffer cells and then secreted into bile. Nevertheless, the healthy biliary tract generally remains sterile and tolerant to pro-inflammatory stimuli of the PAMPs. Indeed, PAMPs may be neutralized by alkaline phosphatase activity. Also, β-defensins are secreted by dedicated PAMPs receptors like Toll-like receptors[45]. FXR appears to be involved in maintaining sterility of the biliary tract: FXR activation by bile acids facilitates vasoactive intestinal peptide receptor-1 (VPAC1)-induced choleresis[46], promotes expression of α-crystalline (a putative defence molecule against oxidative stress) and induces –in cooperation with activated Vitamin D receptor- expression of the antimicrobial peptide cathelicidin in biliary epithelial cells[45,47]. These processes appear deficient in primary biliary cirrhosis and primary sclerosing cholangitis, with the result that PAMPs accumulate in the biliary tract and induce inflammation[45]. The potent synthetic FXR agonist 6-ethyl chenodeoxycholic acid (6-ECDCA) is currently evaluated in clinical trials of patients with primary biliary cirrhosis. Nevertheless, it is important to realize that in cholestasis with an obstructive component and in extrahepatic cholestasis, increased hepatic bile acid secretion could in theory be detrimental for the biliary system, leading to higher biliary pressure and rupture of cholangiocytes[48,49].

3.2 Cholesterol gallbladder stones.

Gallstones are a major health burden in the Western world, approximately 10-15% of the entire population being afflicted. Cholesterol gallstones are aggregates of cholesterol crystals phase-separated from cholesterol-supersaturated bile (see Section 2.1). FXR-ko mice on lithogenic diet are highly susceptible to cholesterol gallstone formation, with biliary cholesterol supersaturation and marked gallbladder wall inflammation. The explanation is that relative amounts of solubilizing bile acids and phosphatidylcholine in bile are decreased, since transport proteins ABCB11 and ABCB4 are less expressed in absence of FXR. In wild type (WT) mice on lithogenic diet, gallstone formation can be prevented by the synthetic FXR agonist GW4064, because increased amounts of solubilizing bile acids and phospholipids prevent cholesterol supersaturation and nucleation of cholesterol crystals under these circumstances[50]. Data on role of FXR in human cholesterol gallstone formation are limited. In the subgroup of female non-obese gallstone patients, decreased enterocytic

expression of FXR and its target genes ASBT, ileal lipid binding protein (ILBP) and OSTα-OSTβ (all involved in bile acid transport) has been described[51,52]. These findings suggest an intestinal defect with decreased absorption and subsequently diminished bile acid pool. In line with these data, increased bile acid and cholesterol synthesis have been reported in a subgroup of Chilean patients from Amerindian descent[53]. This defect was supposed to be secondary to increased intestinal loss of bile acids, and preceded actual gallstone formation. Data on FXR gene polymorphisms have revealed controversial results: in a Mexican population, the most common haplotype NR1H4_1 was associated with gallstone prevalence; in contrast, NR1H4_1 displayed no association with gallstone prevalence in a German population, whereas in a Chilean population a trend towards a protective effect of NR1H4_1 was observed[54].

3.3 Hepatic inflammation, fibrosis and metabolic syndrome.

Hepatic inflammation is a crucial characteristic of many liver diseases such as viral hepatitis, autoimmune hepatitis and non-alcoholic steatohepatitis. Recent evidence points to a novel role of FXR in regulating the hepatic inflammatory response.

3.3.1 Animal data.

FXR-ko mice display pronounced inflammation and elevated expression of inflammatory genes in the liver[55]. After lipopolysaccharide (LPS) administration, the livers of FXR-ko mice reveal massive necrosis and inflammation with infiltration of inflammatory cells, accompanied by higher expression levels of inflammatory markers (iNOs, COX-2, IP-10, IFNγ) which is not the case in WT mice[56]. Furthermore, mice which expressed constitutively active FXR in their livers after adenoviral VP16-FXR tail injection exhibit decreased expression of inflammatory markers after LPS injection[56]. *In vitro*, induction of pro-inflammatory iNOS, IL-1α and IL-6 in response to TNFα or LPS was higher in primary hepatocytes from FXR-ko than from WT mice, and could be inhibited by the FXR ligands GW4064 or 6-ECDCA only in primary hepatocytes from WT mice.

3.3.2 Non-alcoholic fatty liver disease.

One of the most important liver diseases in the Western world is non-

alcoholic fatty liver disease (NAFL), present in up to 30% of all Americans. In this condition, liver steatosis occurs, related to obesity, metabolic syndrome, insulin resistance and type 2 diabetes mellitus. Progression to fibrosis and cirrhosis may occur if there is associated inflammation in the liver: non-alcoholic steatohepatitis (NASH). Up to one third of subjects with NAFL may exhibit NASH on liver biopsy. In up to 10% of all liver transplants in the Western world, NASH is the underlying cause of the liver disease[57]. As indicated in Figure 2, FXR activation exerts various potentially beneficial effects on triglyceride and glucose metabolism, which may improve NAFL. The Zucker (fa/fa) rat is a model for NAFL, since liver steatosis, diabetes, insulin resistance and obesity occur due to a loss-of-function mutation of the leptin receptor. FXR activation by 6-ECDCA protects against liver steatosis, body weight gain, and muscle fat deposition and reverses insulin resistance in this model[58]. Preliminary data in humans with NAFL and diabetes also indicate improved liver biochemistry and better glycemic control during short term therapy with 6-ECDCA[59]. Evidence obtained from experimental animal models suggests that FXR inhibits inflammation and fibrosis in NAFL: in the low-density lipoprotein receptor-ko (LDLr-/-) hypercholesterolemic mouse on high fat diet, macrosteatosis and hepatocyte ballooning occur, without inflammation. In contrast, in the liver of the LDLr-ko/FXR-ko double-ko mouse, marked foci of inflammatory infiltrate are present under these conditions, with increased hepatic mRNA levels not only of *TNFα*, but also of procollagen1α1 and TGF-β and type 1 collagen protein levels, as markers for liver fibrosis[60]. In mice on methionine and choline deficient diet (another model for NASH), the FXR agonist WAY-362450 reduces liver inflammation and fibrosis, without impact on triglyceride accumulation[61]. Similar effects were found in the ConA model of murine acute hepatitis[62]. Of note, apart from their anti-inflammatory effects and effects on lipid and glucose metabolism, FXR agonists may also inhibit fibrosis progression by inhibiting hepatic stellate cell activation (a key event in progression to liver fibrosis and cirrhosis), supposedly through FXR-PPARγ-dependent or FXR-SHP dependent mechanisms[63,64]. Nevertheless, the relevance of these findings for human disease is controversial, since in some mouse models, FXR ablation is associated with decreased rather than increased fibrosis, and FXR protein could not be detected in human hepatic stellate cells or portal human myofibroblasts[65].

3.4 Liver regeneration.

Liver regeneration is an important topic in current research. Progress in hepatobiliary surgery allows extensive liver resections in patients with hepatocellular carcinoma or metastatic liver disease. Nevertheless, regenerative capacity of the remnant liver is the limiting factor for the extent of the resection. Regeneration is a fundamental response after various forms of injury. This process consists of several well-orchestrated phases, with rapid induction of proliferative factors activating the quiescent hepatocytes and priming their subsequent proliferation, followed by renewed quiescence. Important early events include the secretion by hepatocytes of growth factors and cytokines such as Transforming Growth Factor (TGF)α, as well as the secretion by nonparenchymal cells of IL-6 and TNFα. Activation of these signalling pathways increases the expression of many target genes by activating transcription factors, including Stat3, NF-κB, and c-Myc. Partial hepatectomy is a frequently used model to study liver regeneration. The observation that in rats whose circulatory systems are linked by parabiosis, partial hepatectomy of one animal induces hepatocyte proliferation in the other, indicates the presence of a circulating "regenerative stimulus". Nevertheless, the mechanism by which hepatocytes sense the loss of functional capacity and the identity of this stimulus has remained elusive for a long time. Recent research points to bile acids and FXR as attractive candidates. In the early phase of partial hepatectomy, exposure of remaining hepatocytes to bile acids is increased, with maintained expression levels of canalicular transporters and bile flow. Huang *et al.*[66] demonstrated in WT mice, that feeding 2% cholic acid enhanced, and feeding the bile acid-binding resin cholestyramine inhibited liver regeneration after partial hepatectomy. Also, FXR-ko exhibited defective liver regrowth, which was not affected by bile acid enrichment or depletion. Similarly, in pregnant FXR-ko mice, normal pregnancy-induced liver growth is altered, with adaptive hepatocyte hyperplasia[67]. Forkhead box m1b (Foxm1b) -a general proliferative transcription factor involved in cell cycle regulation and required for liver regeneration- was recently identified as an FXR target gene[66,68]. Of note, ligand-induced FXR activation induces Foxm1b expression and improves the defective regeneration in aging livers.[68] These findings suggest FXR activation as a potential new approach to promote liver regeneration in elderly subjects, or after liver resection (especially

in case of compromised liver reserve due to preexistent hepatic disease).

3.5 Hepatocellular carcinoma

Hepatocellular carcinoma is a frequent complication in patients with cirrhosis due to inflammatory liver diseases. In FXR-ko mice, pronounced inflammation and elevated expression of inflammatory genes in the liver are associated with spontaneous tumor formation[55]. Of note, similar to human hepatocellular carcinoma, preliminary data indicate activation of the Wnt-β-catenin pathway in the earliest stages of tumor formation in FXR-ko mice, even before tumors have formed[69]. This phenomenon may occur in part secondary to stimulation of the canonical Wnt pathway, as suggested by increased inactivation of GSK-3β (ser9 phosphorylation), increased disheveled expression and decrease in Wnt antagonist sFRP. Similarly, a substantial decrease in E-cadherin accompanied by a substantial decrease in its negative regulator snail was observed in FXR-ko mice[69]. Further research on the role of FXR in human hepatocellular carcinoma and in murine models should be performed, before clinical trials with FXR agonists can be proposed in these patients.

4. FXR and the gastro- intestinal tract

4.1 Inflammatory bowel disease.

Recently, FXR has been implicated in pathogenesis of idiopathic inflammatory bowel disease. This entity comprises two types of chronic intestinal disorders: Crohn's disease and ulcerative colitis. Accumulating evidence suggests that these diseases result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host. Genetic data indicate the importance of host-microbe interactions in pathogenesis of these diseases and various genes have been associated with inflammatory bowel disease (especially Crohn's disease), including: 1. The nucleotide oligomerization domain 2 (NOD2) gene which encodes a protein that acts as an intracellular sensor of bacterial peptidoglycan; 2. autophagy genes like ATG16L1 (autophagy-related 16-like 1) which enable cells to regulate and degrade various intracellular components, including pathogens; and 3. the interleukin 23-Th17 pathway which mediates antimicrobial defense and intestinal inflammation. Nevertheless, it is estimated that known genetic associations account for

only 20% of the genetic variance underlying susceptibility to inflammatory bowel disease, leaving ample room for additional genetic factors[70]. It is well known, that intestinal bile acid deficiency is associated with bacterial overgrowth and compromised barrier function, which can be reversed by oral bile acid feeding. These findings cannot be explained by direct antibacterial effects of bile acids[71]. Recently, the bile acid-FXR axis has been implicated in intestinal barrier function and antibacterial defense: Inagaki *et al.*[72] found bacterial overgrowth, increased intestinal permeability and large amounts of bacteria in mesenteric lymphonodes in FXR-ko mice. Bile duct ligation markedly aggravated these abnormalities. Of note, there was also significant inflammation of the intestinal wall. Furthermore, FXR activation by the synthetic ligand GW4064 offered strong protection against these adverse events in WT, but not in FXR-ko mice. Interestingly, the FXR ligand increased mRNA expression of *Inducible Nitric Oxide Synthase* (*iNOs*), *Angiogenin 1* (*Ang1*) and *Carbonic anhydrase 12* (Car12) (see Figure 2). These genes are involved in antibacterial defence, by producing antimicrobial peptides (iNOs and Ang1) or maintaining appropriate intestinal pH (Car12), important for the homeostasis of intestinal luminal contents and epithelial barrier integrity. Vavassori *et al.*[73] recently provided support for involvement of FXR in inflammatory bowel disease: in the basal state, significant inflammation and pro-inflammatory cytokine mRNA expression were detected in 16-weeks old FXR-ko mice. In two complementary experimental murine models (intrarectal administration of trinitrobenzensulfonic acid and oral administration of dextrane sodium sulfate), concurrent administration of the potent synthetic FXR ligand 6-ECDCA protected against the colitis in WT mice, (with reduced expression of various pro-inflammatory cytokines in colonic homogenates and colon-derived macrophages), but not in FXR-ko mice. Both in murine models and in Crohn's disease patients, intestinal inflammation was associated with decreased *FXR* mRNA expression.

4.2 Colonic cancer.

In the Western world, colon cancer is the second leading cause of death from cancer in adults. The pathogenesis of colon cancer is a multistep process with several mutations (including mutations in the APC, adenomatous polyposis coli gene) during the adenoma-carcinoma sequence. Various nutrients, such as high fat diet may modify the progression of the disease, possibly

through increasing the amount of bile acids in the colon. Of note, chronic inflammation as occurs in idiopathic inflammatory bowel disease is associated with considerably enhanced risk of tumour formation. There is considerable evidence for a role of FXR in modulating the course of the disease. First, *FXR* mRNA expression is decreased in colonic polyps, and even more pronounced, in colonic adenocarcinoma[74]. Second, in two experimental murine models for intestinal cancer (administration of the carcinogen azoxymethane and APCmin mice), FXR deficiency led to significantly increased sizes and numbers of the tumors[75]. Third, Modica *et al.*[76] found in 8-week old FXR-ko-APC+/- mice, adenomatous-like lesions and numerous aberrant crypt foci (the latter considered the earliest stages of tumorigenesis), even before carcinomas actually had formed. At this stage, there was extensive mucosal infiltration of neutrophils and macrophages in the mucosa of FXR-ko-APC+/- mice, along with increased *TNFα* mRNA expression, nuclear β-catenin accumulation and Ki-67 positive cells. Of note, TNFα is able to promote Wnt-β-catenin signaling and tumor development, and TNFα blocking has been shown to reduce intestinal tumors in the azoxymethane-dextrane sodium sulphate (AOM-DSS) model of colon carcinogenesis associated with chronic colitis[77]. FXR-ko mice exhibit a markedly expanded bile acid pool, since negative feedback regulation of bile acid neosynthesis by the FXR-FGF15 axis is absent. One might therefore argue, that the high susceptibility of FXR-ko-APC+/- mice to intestinal tumorigenesis might relate to the elevated bile acid levels per se rather than to a direct effect of the intestinal FXR loss. The fact that bile acid depletion with the resin cholestyramine did not affect numbers of aberrant crypt foci, argues against this explanation[76]. Also, similar findings were obtained in the AOM-DSS model, again without effects of bile acid depletion by cholestyramine. Finally, in a xenograft mouse model, activating FXR with AdVP16FXR led to markedly increased apoptosis and significantly reduced tumor growth[76].

4.3 Esophageal intestinal metaplasia and cancer.

Esophageal reflux disease is very common in the Western world. This condition may cause chronic inflammation in the distal esophagus and replacement of the normal squamous epithelium by specialized intestinal epithelium, so called Barrett's esophagus. This premalignant condition can lead to development of adenocarcinoma. Combined pathologic bile acid and acid exposure is a prerequisite for the development of Barrett's esophagus[78,79]. The transcription

factor NF-κB has been implicated in bile acid-induced inflammation in Barrett's esophagus[80,81]: in esophageal cell cultures, especially bile acids are able to activate NF-κB[80]. Resulting inflammatory changes may promote dysplasia and cancer, e.g. through increased cell proliferation, escape of apoptosis, loss of cell-cell control and cell-cell adhesion. Indeed, in cultures of esophageal cells, additon of bile acids to the medium induces expression of cancer-related gene products such as *p53*, *c-myc*, *cyclo-oxygenase* (*COX*)*2*, *CDX-1* and *CDX-2* (caudal related homeobox gene family: intestine specific transcription factors that are important in early differentiation and maintenance of intestinal epithelial cells), similar to findings in patients with Barrett's esophagus and in animal models for this condition[81,82]. In the squamous epithelium lining the normal esophagus, expression of *FXR* and its target genes is very low or even absent. In contrast, *FXR* is markedly expressed in reflux esophagitis, and even more in Barrett's esophagus without dysplasia[83,84]. There is a concomitant increase in expression of pro-inflammatory *IL-8* and *macrophage inflammatory protein 3α* (*MIP3α*). Similar increased mRNA expression of *FXR*, *IL-8* and *MIP3α* has been found *in vitro* in the esophageal cell line TE7 after exposure to deoxycholic acid. Pretreatment with the FXR antagonist guggulsterone inhibits the increased expression under these circumstances[83]. Also, Dvorak *et al.* recently found increased expression (at mRNA and protein level) of FXR-dependent bile acid transport proteins ASBT (at the apical membrane) and IBABP (cytoplasmic localization) in non-dysplastic Barrett's esophagus, with progressive loss at increasing degree of dysplasia[85]. Based on these data, the authors hypothesized, that FXR-dependent bile acid transporters remove excess bile acids from the esophageal lumen. Decline in transporter expression that occurs in progression of nondysplastic Barrett's esophagus to early adenocarcinoma could then be a further adaptation to high bile acid exposure in order to limit DNA damage to the metaplastic cells[85]. However, this hypothesis needs to be studied further as well as the mechanism by which expression of these bile acid transporters is reduced during progression from nondysplastic Barrett's esophagus to early adenocarcinoma.

5. SUMMARY AND PERSPECTIVE

In this review we have discussed the role of the bile acid-FXR axis in the patho-physiology of the liver, the biliary and the gastrointestinal tract. Initially

identified as master regulator of bile acid homeostasis, known FXR functions now range from control of cholesterol, lipid and glucose metabolism to its intriguing role in inflammation, liver regeneration and inflammation-related carcinogenesis. Limiting the extent of the inflammatory response by inhibition of the transcription factor NF-κB appears a central theme in this respect. In principle, inflammation is considered a beneficial response to injury or infection, to remove the harmful stimulus and to initiate the healing process. However, when uncontrolled, inflammation may lead to chronic inflammatory disease and increased cancer risk. In the current review, we have discussed chronic inflammatory diseases of the liver, the intestine and the esophagus, as well as inflammation-related carcinogenesis in these organs as striking examples. Activated NF-κB down-regulates the transcriptional activity of many steroid and nuclear receptors. For example, it has been shown that the nuclear steroid and xenobiotic receptor SXR and NF-κB suppress each other[86]. Recent complementary *in vitro* and *in vivo* data indicate a similar reciprocal inhibiting interaction between NF-κB and FXR in hepatic inflammation[56,87]. Despite the described involvement of FXR activity in counter-regulating the inflammatory response, the exact underlying mechanisms remain to be explored in detail. FXR-dependent inhibition of the NF-κB pathway due to decreased binding between NF-κB itself and its own responsive element on the DNA sequences has been suggested, based on limited *in vitro* experiments[56]. There is also some evidence for inhibition by FXR of nuclear co-repressor clearance on the NF-κB responsive element on the promotor[73]. Nevertheless, analogous to other nuclear receptors such as the Glucocorticoid Receptor, the Liver X Receptor or the Peroxisome Proliferator Activated Receptor, additional mechanisms are well possible for the ability of FXR to antagonize the inflammatory response (see Figure 3). NF-κB trans-repression consists of a spectrum of molecular mechanisms by which nuclear receptors can inhibit signal-dependent gene activation with or without direct, sequence-specific binding to DNA[88]. These mechanisms vary from increased transactivation of IκBα (the cytosolic NF-κB inhibitory protein) to direct binding of nuclear receptors to NF-κB in the nucleus with the result that binding of co-activator to NF-κB is prevented because of sterical reasons. Furthermore, post-translational modification of the nuclear receptor (e.g. sumoylation) can inhibit the inflammatory responses by blocking the clearance of the co-repressor complexes from the NF-κB complex. Also, the

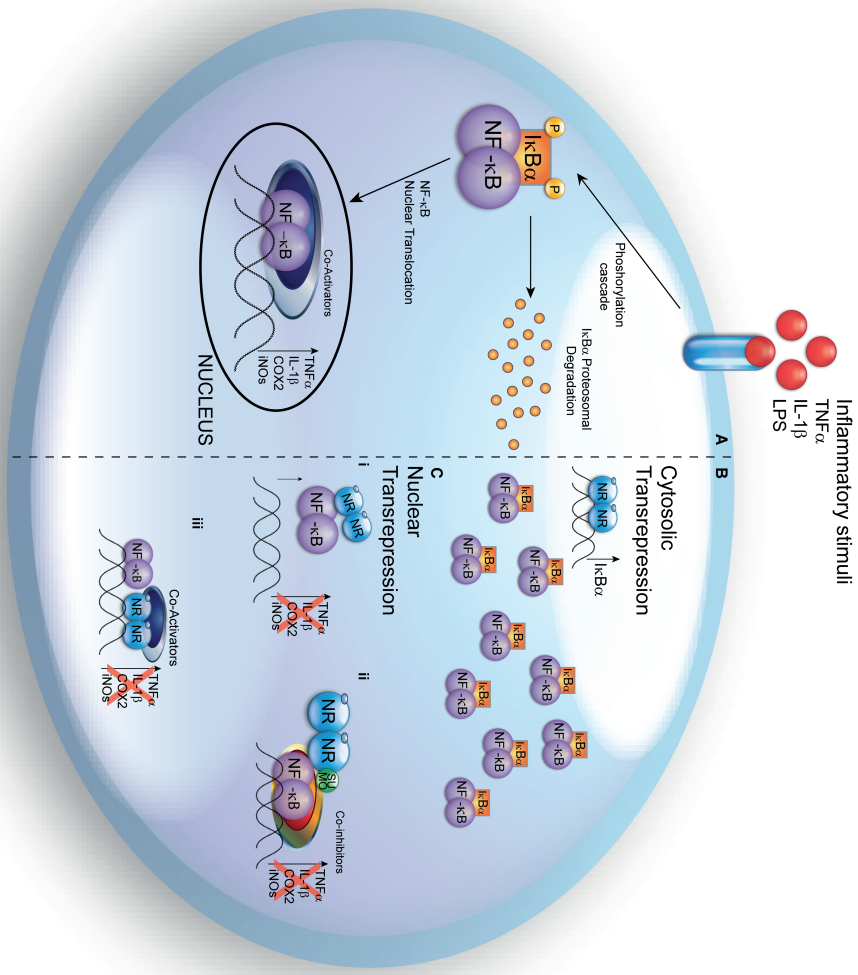

Figure 3: *NF-κB pathway and its inhibition by nuclear receptor transrepression.* **Left side of Figure:** A. In the absence of an inflammatory stimulus, NF-κB is retained in the cytoplasm, bound to the NF-κB inhibitor IκBα, which prevents NF-κB nuclear translocation. In presence of an inflammatory stimulus (e.g. TNFα, IL-1β, LPS) a phosphorylation cascade occurs, ultimately resulting in IκBα phosphorylation. This event targets IκBα to proteosomal degradation and makes NF-κB complex free to translocate

to the nucleus. Here NF-κB binds to κB Responsive Elements and initiates transcriptions of genes involved in the inflammatory response, such as TNFα, IL-1β, COX2 and iNOs. **Right side of Figure:** Nuclear receptors mediate NF-κB transrepression through various mechanism and at different levels.
B. Cytosolic transrepression: nuclear receptors may increase the expression of IκBα, thereby increasing the retention of NF-κB complex in the cytosol.
C. Nuclear transrepression: inhibition of NF-κB target gene transcription can be achieved by: i Direct binding of nuclear receptors to the NF-κB complex. In this way the binding of the co-activator to NF-κB is prevented because of sterical reasons. ii. Post-translational modification of nuclear receptors (e.g. Sumoylation). This prevents the co-repressor complex clearance from NF-κB. iii Cofactor squelching in which competion for a limited pool of coactivators between NRs and NF-κB is in favour of the nuclear receptor.

two different transcription factor complexes can compete for the same pool of coactivators, with the “cofactor squelching” mechanism. Future research should be aimed to elucidate how FXR regulates the inflammatory process, at what point in the inflammatory response timeline, and how this will affect metabolic homeostasis. There is a distinct possibility that modulation of FXR expression may be transferred from bench to bedside, by using this nuclear receptor as a drug target for various diseases of the hepatobiliary and gastrointestinal tract. FXR might be of crucial importance for the development of new therapeutic strategies targeting the “bad”, preserving the “good”, to modulate its anti-inflammatory and anti-tumour action without altering its function in the metabolic control. The challenge for the immediate future will be to functionally dissect the transcriptional functions (transactivation and transrepression) of FXR. Only then will it be possible to develop FXR-modulators as therapeutic approaches without causing undesired side effects. In the meantime results of currently performed studies with synthetic FXR agonists in patients with metabolic and liver disease are eagerly awaited.

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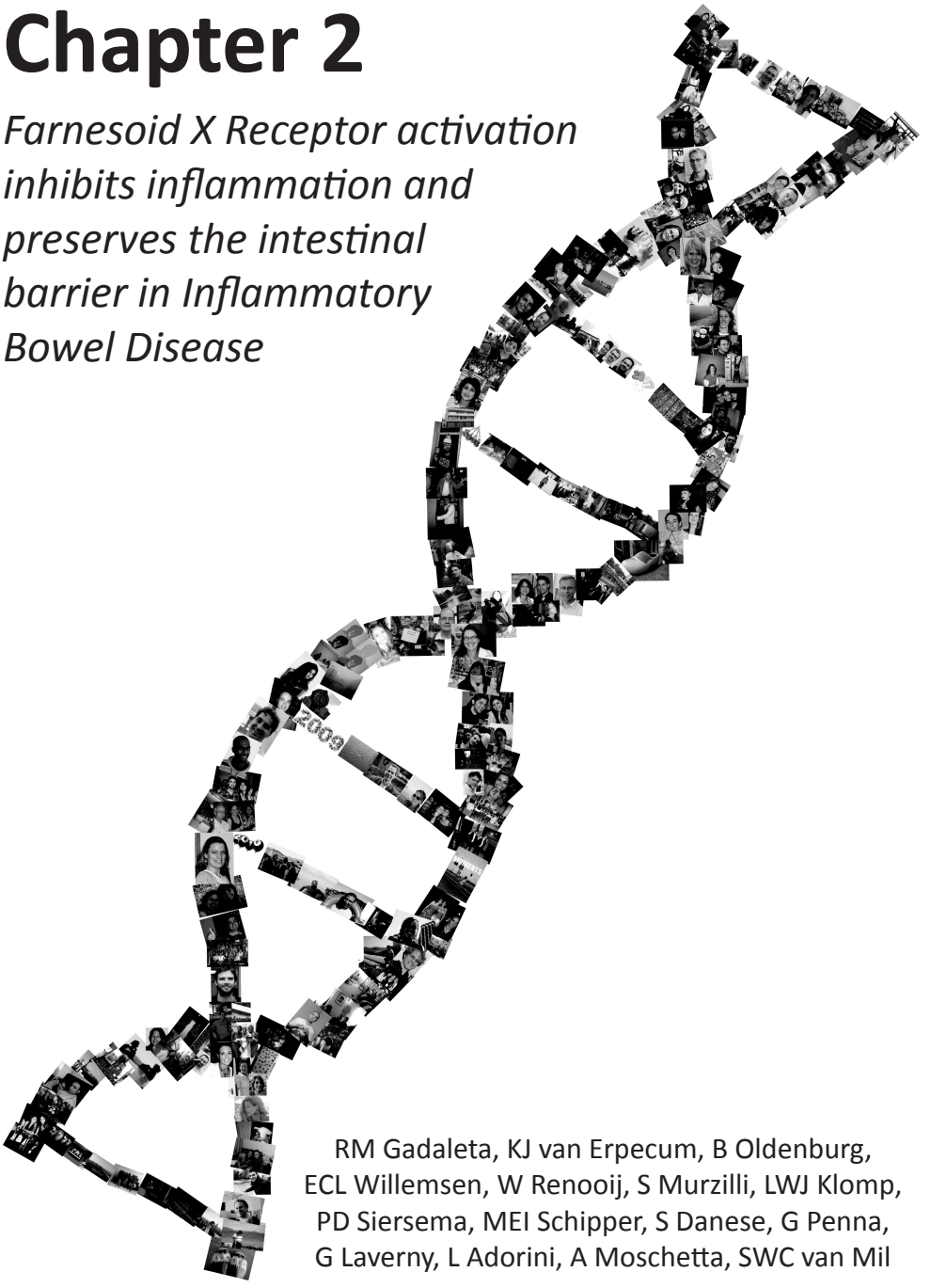
Chapter 2

Farnesoid X Receptor activation inhibits inflammation and preserves the intestinal barrier in Inflammatory Bowel Disease



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Gut. April 2011

ABSTRACT

Background & aims: Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation, resulting from dysregulation of the mucosal immune system and compromised intestinal epithelial barrier function. The bile salt nuclear Farnesoid X Receptor (FXR) was recently implicated in intestinal antibacterial defence and barrier function. We investigated the therapeutic potential of FXR agonists for treatment of intestinal inflammation in complementary *in vivo* and *in vitro* models. ***Methods:*** Colitis was induced in wild type (WT) and Fxr-ko mice using DSS and in WT mice using TNBS. Mice were treated with vehicle or with the FXR agonist INT-747 and colitis symptoms were assessed daily. Epithelial permeability assays and cytokine expression analysis were conducted in mouse colon and in enterocyte-like cells (Caco-2/HT29) treated with medium or with INT-747. Inflammatory cytokine secretion was determined by ELISA in various human immune cell types. ***Results:*** INT-747-treated WT mice are protected from DSS- and TNBS-induced colitis, as shown by significant reduction of body weight loss, epithelial permeability, rectal bleeding, colonic shortening, ulceration, inflammatory cell infiltration, and goblet cell loss. Furthermore, FXR activation in intestines of WT mice and in differentiated enterocyte-like cells downregulates expression of key pro-inflammatory cytokines and preserves epithelial barrier function. INT-747 significantly decreases TNFα secretion in activated human peripheral blood mononuclear cells (PBMCs), purified CD14+ monocytes and dendritic cells, as well as in lamina propria mononuclear cells (LPMCs) from IBD patients. ***Conclusions:*** FXR activation prevents chemically-induced intestinal inflammation with improvement of colitis symptoms, inhibition of epithelial permeability and reduced goblet cell loss. Furthermore, FXR activation inhibits pro-inflammatory cytokine production *in vivo* in the mouse colonic mucosa, and *ex vivo* in different immune cell populations. Our findings provide a rationale to explore FXR agonists as a novel therapeutic strategy for IBD.

Summary box:

What is already known about this subject?

- Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation, resulting from dysregulation of the mucosal immune system and compromised intestinal epithelial barrier function.
- Bile duct obstruction in humans and mice is associated with mucosal injury, bacterial overgrowth and translocation. Oral administration of bile salts or FXR agonists counteracts these deleterious effects.
- Fxr activation protects against inflammation presumably by repression of NF-κB signalling in mice.
- Fxr activation counter-regulates inflammatory cytokine expression in activated murine and human immortalized immune cells.

What are the new findings?

FXR activation ameliorates intestinal inflammation at multiple levels:

- DSS- and TNBS-induced colitis in mice results in significant goblet cell loss, and Fxr activation partially protects from the decrease in goblet cells.
- FXR activation preserves the integrity of the intestinal epithelial barrier.
- FXR activation counteracts NF-κB-mediated immune responses in enterocytes *in vitro*.
- FXR activation inhibits inflammatory signalling in various primary human immune cell types (PBMCs, CD14+ monocytes and monocyte-derived dendritic cells). Moreover, the anti-inflammatory properties of FXR activation are confirmed *ex vivo* in lamina propria mononuclear cells (LPMCs) from IBD patients.

How might it impact on clinical practice in the foreseeable future?

This multi-level protection against intestinal inflammation provides a rationale to explore FXR agonists as a novel therapeutic strategy for IBD in humans.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation, with potentially severe complications and even mortality[1]. Although the exact aetiology is unclear, it is thought to result from a combination of dysregulation of the mucosal immune system and compromised intestinal epithelial barrier function in genetically predisposed individuals[2]. Several genes associated with IBD are involved in anti-bacterial defence (e.g. NOD2, ATG16L1, cathelicidin, defensins) as well as in barrier function (e.g. PTPsigma, MAGI2, myosin IXB and E-cadherin)[3-6]. Current treatment options for IBD are mainly aimed at suppressing the immune response. However, although reasonably effective, significant side effects and treatment failures can occur, stressing the need for novel treatment options in IBD. The intestinal epithelial barrier protects the host by preventing external antigens and micro-organisms from entering the body[7]. This physical barrier consists of enterocytes tightly connected via intercellular junctions. Enterocytes also secrete cytokines and chemokines that trigger the inflammatory response, as a second line of defence against luminal contents. In addition, mucin-secreting goblet cells form the mucus layer that protects the mucosal surface from antigens and contribute in maintaining the intestinal barrier function. IBD patients with clinically and endoscopically significant colitis display various degrees of goblet cell loss[8]. The epithelial barrier is often compromised already in early stages of the disease, leading to bacterial translocation and inflammation[9,10]. Dysregulation of the immune response to intestinal bacteria in IBD patients occurs due to a shift of balance from secretion of anti-inflammatory mediators towards pro-inflammatory molecules. Activation of the Nuclear transcription Factor kappa B (NF-κB) was identified as a key factors in this shift, resulting in strongly enhanced expression of pro-inflammatory genes, and recruitment of excess inflammatory cells to the intestinal wall. Recently, the nuclear Farnesoid X Receptor (FXR) has been implicated in immune modulation and barrier function in the intestine[11]. FXR is activated by bile salts and regulates transcription of genes involved in bile salt synthesis, transport and metabolism in the liver and intestine by binding FXR responsive elements (FXRE) in promoters of target genes as a heterodimer with Retinoid X Receptor (RXR)[12]. In the rodent bile duct

ligation (BDL) model and in humans with bile duct obstruction, bile salt deficiency in the intestine is associated with mucosal injury, bacterial overgrowth and translocation. Strikingly, oral administration of bile salts or Fxr agonists counteracted these deleterious effects[13-16]. Fxr-ko mice exhibited compromised intestinal integrity at baseline, with further deterioration after BDL, which was not prevented by Fxr agonist treatment[15]. Recently, Fxr activation was also shown to protect against hepatic inflammation presumably by repression of NF-κB signalling in mice[17]. In addition, FXR is expressed and counter-regulates inflammatory cytokine expression in activated immune cells[18,19]. Thus, immune cell modulation by FXR activation may be responsible for the improvement of intestinal inflammation[20]. Here, we have applied an integrated *in vitro* and *in vivo* approach to investigate the therapeutic potential of FXR agonists for the treatment of intestinal inflammation. We specifically focused on the crosstalk between the epithelial barrier and immune modulation by enterocytes as well as immune cells. We show that treatment with FXR agonists ameliorates intestinal permeability *in vivo* and *in vitro*, and provide evidence that FXR activation counteracts pro-inflammatory cytokine expression and secretion by enterocytes and by different human immune cell types. The anti-inflammatory properties of the selective FXR agonist INT-747 were also observed in *ex vivo* lamina propria mononuclear cells (LPMCs) from IBD patients.

METHODS

Animals.

9-12 weeks old wild type (WT) C57BL/6J mice were obtained from Charles River Laboratories. Pure strain C57BL/6J Fxr-ko mice (Dr. D.J. Mangelsdorf (Southwestern Medical Center, Dallas, TX) were kindly provided by Dr. Frank Gonzalez (NIH, Bethesda, MD). Mice were fed *ad libitum* and housed in a temperature- and light-controlled room. All experiments were approved by the Ethical Committee of the Consorzio Mario Negri Sud.

FXR pharmacological activation and colitis induction.

Pharmacological activation of Fxr was accomplished by daily oral gavage with 5mg/kg/day INT-747 (6-ethyl-chenodeoxycholic acid, Intercept

Pharmaceuticals Inc, New York, NY)[21] or vehicle (1% Methyl-cellulose) from 3 days prior to chemically-induced colitis treatments, and continued until the end of the experiments. Two well established models for colitis were used. Firstly, colitis was induced by administration of 2.5% (wt/vol) Dextran Sodium Sulphate (DSS; MW 36-50 kDa, MP Biochemicals Inc., The Netherlands) in drinking water for 7 or 10 days (n=8-10 mice per group). Secondly, colitis was induced by two rectal administrations (rectal silicon catheter 3.5 French, Instech Solomon Scientific, San Antonio, TX) of 1.5 mg Trinitrobenzene Sulphonic Acid (TNBS) in 40% ethanol, with a time interval of 7 days. Mice (n=5-8 mice per group) were sacrificed 48h after the second TNBS administration. Daily changes in body weight were assessed. Rectal bleeding was scored on a scale from 0 to 5, indicating no (0) to highly severe (5) rectal bleeding. For intestinal permeability assays, mice were sacrificed after 7 days of DSS treatment or 48 hours after the second TNBS administration. Ilea and colons were snap-frozen or fixed in 10% formalin (24h) and embedded in paraffin.

mRNA extraction and qRT-PCR analysis.
RNA was isolated from ileum and colon of mice using RNeasy Micro kit (Qiagen, Venlo, The Netherlands). RNA was isolated from HT29 and Caco-2 cells using TRIzol reagent. cDNA was generated from 500ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis was carried out using SYBR green PCR mastermix and analysed on a MyIQ real time PCR cycler (Biorad, Veenendaal, The Netherlands). Values were normalized to GAPDH. Primers are listed in Supplementary Table 1.

Bile salt measurements.
Conjugated bile salt species were analyzed by HPLC (22) using a C-18 Waters Bondapack 10µm column (Waters, Milford MA). Methanol/ phosphate buffer was used as the eluent (pH 5.2, flow rate 1ml/min).

Histology.
Distal colon sections (5µm) were stained with hematoxylin/eosin. Histopathological scoring was performed by an experienced pathologist blinded to the experimental condition, employing an established semi-quantitative score ranging from 0 to 6 based on infiltration of inflammatory cells and epithelial damage (1=few inflammatory cells, no epithelial degeneration;

2=mild inflammation, few signs of epithelial degeneration; 3=moderate inflammation, few epithelial ulcerations; 4=moderate to severe inflammation, ulcerations in 25-50% of the tissue section; 5=moderate to severe inflammation, large ulcerations in >50% of the tissue section; 6=severe inflammation and ulcerations of >75% of the tissue section)[23]. Furthermore, depletion of goblet cells was scored using a scoring index from 0 to 4 (0=no depletion; 1=0-10% depletion; 2=10-25%; 3=25-50% depletion; 4=50-100% depletion).

In vivo intestinal permeability assay.
Intestinal permeability was examined in mice following 7 days DSS treatment or 48h after the second TNBS administration, as described previously[24]. Results were normalized to WT mice which did not receive any treatment (n=3). Mice were gavaged with 0.6mg/g body weight of FITC-conjugated dextran (Sigma, MW 3-5 kDa) for 4h. Blood was collected and FITC concentrations were measured in plasma (Fluorimeter Pharos FX, Biorad Hercules, CA).

Permeability and LDH release assays in Caco-2 cell monolayers
Caco-2 cells were grown on collagen-treated PTFE Transwell® filter inserts (pore size=0.4µm, Corning Inc, NY, USA), differentiated for 14 days and incubated with or without 2.5% DSS for 10 days, in presence or absence of 1µM FXR synthetic ligand GW4064 (started 3 days prior to DSS treatment). At day 6, 7, 8, 9 and 10, medium containing 100µM of cell impermeable Lucifer Yellow (Sigma, Saint Louis, Missouri, USA) was added to the apical compartment. After 1.5h, fluorescence was measured in basolateral medium (FLUOstar Galaxy microplate reader, BMG LabTech, Offenburg, Germany). The flux of the Lucifer Yellow into the basolateral compartment was calculated as percentage of total fluorescence applied to the apical compartment. At day 8 of DSS treatment, medium from the apical compartment was harvested for assessment of LDH release (cytotoxicity detection kit, Roche Applied Science, Almere, The Netherlands). Results were normalized to cultures treated with 1% triton-X100 (Sigma) for 10min. At day 8, representative phase-contrast images of the transwells were recorded.

Cell cultures.
HEK293T, HT29 and Caco-2 cells were grown in Dulbecco Modified Eagle's Medium (DMEM) GlutaMax™, supplemented with 10% fetal

calf serum and 1% penicillin/streptomycin (Gibco BRL, Breda, The Netherlands). Caco-2 cells were co-treated with 1% non-essential amino acids. To obtain differentiated HT29 and Caco-2 cells, they were maintained in culture for 7 and 14 days after confluency, respectively.

Reporter assays.
HEK293T cells were co-transfected with a reporter construct containing the responsive element for NF-κB (pGL3-2κB luc) and pcDNA or pcDNA-FXR or pcDNA-FXRW469A (ligand binding domain mutant)[25] together with pcDNA-RXRα using the calcium phosphate method. 24h after transfection, cells were incubated with vehicle (DMSO) and 1μM GW4064 for 24h in the presence or absence of TNFα (500U/ml). Cells were lysed and Firefly and Renilla luciferase activity were measured (Promega Dual-Luciferase Reporter Assay System, Promega, Madison, Wisconsin, USA) with the Centro LB 960 luminometer (Berthold technologies, Vilvoorde, Belgium).

Purification and culture of human immune cells.
Cells were cultured in RPMI 1640 GlutaMax™ culture medium supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (Charles River, Lecco, Italy), 1% non-essential aminoacids, 0.5mg/ml gentamicin and 1mM of sodium pyruvate. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy subjects by ficoll gradient (BioChrome AG, Berlin, Germany), and monocytes were purified from PBMCs by positive selection with CD14 beads (Milteny Biotec, Bergish Gladblach, Germany). PBMCs or monocytes were cultured, as previously described[26] in the presence of 100ng/ml of lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (Sigma-Aldrich, Milano, Italy), with or without 10μM INT-747. Supernatants were harvested after 24h and stored at -80°C until analysis. Monocyte-derived dendritic cells were differentiated from purified monocytes[27] and cultured in complete medium containing 10ng/ml rhGM-CSF and 10ng/ml rhIL-4 (PharMingen, San Diego,CA) in presence or absence of 10μM INT-747. After 7 days, cells were harvested and either stimulated for 24h with 100ng/ml LPS or immediately stained with monoclonal antibodies, anti-CD1a PE (HI149) and anti-CD14 FITC (M5E2) (Pharmingen). Flow cytometric analysis was performed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Lamina propria mononuclear cells

(LPMCs) were isolated from colonic or ileal biopsies of 2 patients with active UC and 2 patients with active CD, as previously described.[28] One patient with UC was under 5-Aminosalicylic acid treatment, while the second was taking azathioprine. One CD patient was taking no medication, while the second was taking azathioprine. Patients gave their informed consent and the study was approved by the Local Ethical Committee. Enriched lamina propria leukocytes were cultured in complete medium supplemented with 100IU/mL penicillin, 0.1mg/mL streptomycin and 0.5μg/mL amphotericin B in presence of 1μg/mL coated anti-CD2 and soluble anti-CD28 (BD biosciences, Milano, Italy). Cells were treated with vehicle or 10μM INT-747 and supernatants were harvested after 24h or 72h as specified in the figure legend, and stored at -80°C until analysis. ELISAs for human interferon γ (IFNγ), TNFα and interleukin 6 (IL-6), were performed using mAb pairs and standards provided by BD OptEIA™ Human ELISA set (BD Biosciences), and myeloperoxidase (MPO) (HBT, Uden, The Netherlands), according to the manufacturer's procedures.

Statistical analysis.

Results are expressed as means ±SEM or ±SD as indicated in the figure legends. Statistical significance was determined by the Student's t test or ANOVA with post-hoc Bonferroni test, as appropriate. All statistical calculations were performed with SPSS software (SPSS Inc., Chicago, Illinois). Two sided p-values *p<0.05 **p<0.01 ***p<0.001 are considered statistically significant.

RESULTS

Oral gavage of INT-747 activates Fxr in ileum and colon and enters the enterohepatic circulation.

To confirm that the dose of INT-747 applied activates Fxr *in vivo* in the intestine, Fxr target gene expression in ileum and colon was assayed. In WT mice, Fxr mRNA expression did not change upon INT-747 treatment. However, Fxr target genes were induced both in ileum (*small heterodimer partner Shp*, 52 fold; *fibroblast growth factor Fgf15*, 4 fold) and colon (*Shp*, 19 fold). Fxr-ko mice expressed very low levels of *Shp* and *Fgf1*5, which did not change upon INT-747 treatment (Supplementary Figure 1).

Taurine-conjugated INT-747 is enriched in bile of WT (9.8±0.8%) and Fxr-ko (5.3±0.8%) mice after oral gavage of INT-747 for 13 days, indicating that INT-747 enters the enterohepatic circulation. In addition, the ratio between tauro-conjugated cholic acid (TCA) and β-muricholic acid (TMCA) is decreased by INT-747 treatment (1.16±0.22 vs. 0.57±0.11), suggesting that Cyp7a1-mediated hepatic cholic acid synthesis is repressed by Fxr activation. Together, these results indicate that INT-747 is retained into the enterohepatic circulation with efficient activation of FXR and its target genes.

Fxr activation by INT-747 decreases symptoms of DSS-induced colitis.
We investigated whether INT-747-mediated Fxr activation confers protection against DSS-induced colitis. INT-747 treatment significantly reduced body weight loss (Figure 1A) and rectal bleeding scores (Figure 1B) and prevented

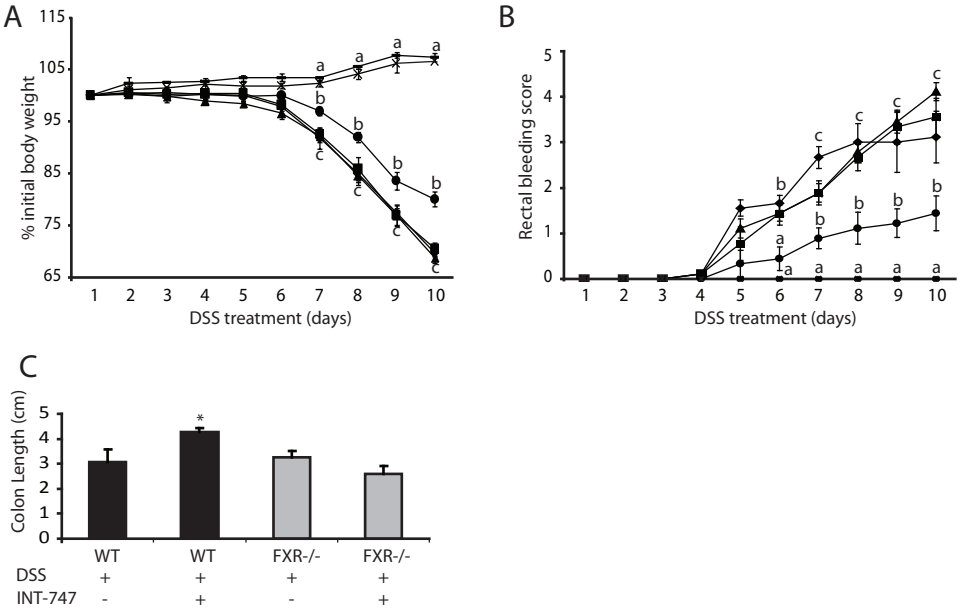

Figure 1: I*NT-747-dependent Fxr activation confers protection against clinical symptoms of DSS-induced colitis.*
(A) Percentage of initial body weight during DSS treatment and (B) Rectal bleeding score; ANOVA statistical analysis with Bonferroni post-hoc test was performed for each time point; significant differences ($p<0.05$) are indicated by different letters. (C) Colon length (cm) of WT (black bars) and Fxr-ko mice (grey bars); Student's t-test, *$p<0.05$, compared to WT DSS vehicle-treated mice. (WT-; Fxr-ko **x**; WT DSS ◆; WT DSS+INT-747 ●; Fxr-ko DSS ▲; Fxr-ko DSS+INT-747 ■)

colonic shortening (Figure 1C) in WT mice (normal colon length 6.5-7 cm, data not shown). In addition, histological analysis showed that DSS-induced colitis was associated with complete disruption of the epithelial layer and acute inflammatory infiltrates in WT and Fxr-ko mice. In sharp contrast, INT-747-treated WT mice showed significantly less morphologic alteration and decreased inflammatory infiltrates. In addition, goblet cell loss due to DSS-induced inflammation was significantly less in INT-747-treated WT mice (Figure 2). INT-747 had no effect on colitis symptoms and histology in Fxr-ko mice.

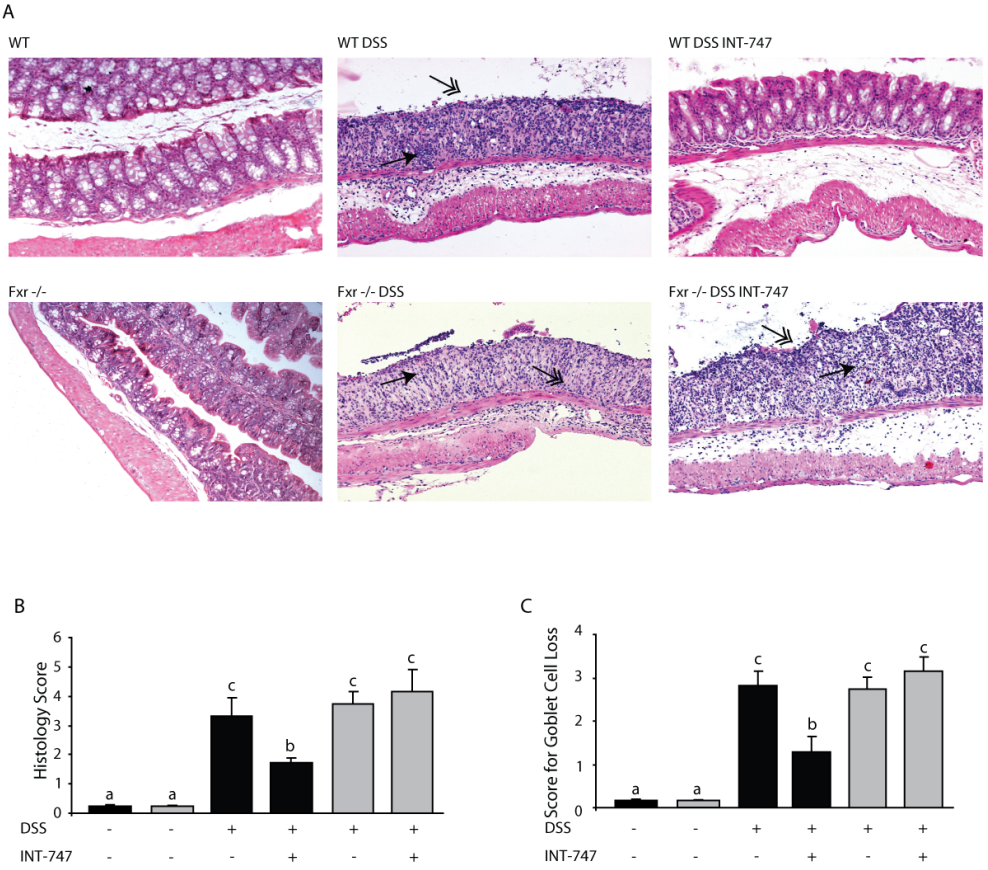

Figure 2: *INT-747-dependent Fxr activation ameliorates histological characteristics of inflammation.*

(A) Hematoxylin and eosin-stained colonic sections. Some DSS-treated WT mice that received INT-747 were almost completely protected from DSS-induced inflammation (upper left panel). Arrows point at inflammatory infiltrates (→) and epithelial degeneration (↦). Magnification 100x. (B) Histology score; (C) Score for goblet cell loss. Each bar

represents the mean value±SEM. ANOVA statistical analysis with Bonferroni post-hoc test was performed, significant differences ($p<0.05$) are indicated by different letters.

In a separate experiment, in which WT mice were treated with DSS for 5 days and were resumed on water until day 10, body weight, rectal bleeding, stool consistency and colon length approached normal values much quicker in INT-747-treated compared to vehicle-treated mice, indicating the capacity of this compound to reduce symptom duration (Supplementary Figure 2A-D). Together, these data indicate that treatment with INT-747 results in significant amelioration of DSS-induced IBD in WT, but not in Fxr-ko mice.

Fxr activation decreases DSS-induced intestinal permeability in vivo and in vitro.

We next investigated whether Fxr activation affects DSS-induced epithelial permeability. Plasma levels of FITC-conjugated dextran were markedly increased in WT and Fxr-ko mice in which DSS-mediated colitis was induced (Figure 3A). INT-747 treatment almost completely abolished DSS-induced permeability in WT but not in Fxr-ko mice. Also, in animals without DSS treatment, intestinal permeability was much lower in WT compared to Fxr-ko mice (Supplementary Figure 3). These data indicate that INT-747-dependent Fxr activation preserves the integrity of the intestinal epithelial barrier.

FXR activation protects against DSS-induced permeability in Caco-2 cell monolayers.

To study the effects of FXR activation on intestinal permeability *in vitro*, we studied apical to basolateral flux of the cell-impermeable Lucifer Yellow compound as a measure for permeability of differentiated Caco-2 cells. Caco-2 cells are enterocyte-like cells that have previously been described in permeability assays[29,30]. FXR is only expressed and functional in fully differentiated Caco-2 cells, comparable to *in vivo* expression[31] (Supplementary Figure 4A and B). Cells treated with 2.5% DSS showed increased permeability of the monolayer starting between days 7 and 8. However, permeability was markedly decreased upon co-treatment with the synthetic FXR agonist GW4064 (Figure 3B), suggesting that FXR activity confers protection against DSS-induced monolayer permeability. In line with this finding, the typical Caco-2 cell monolayer morphology changed

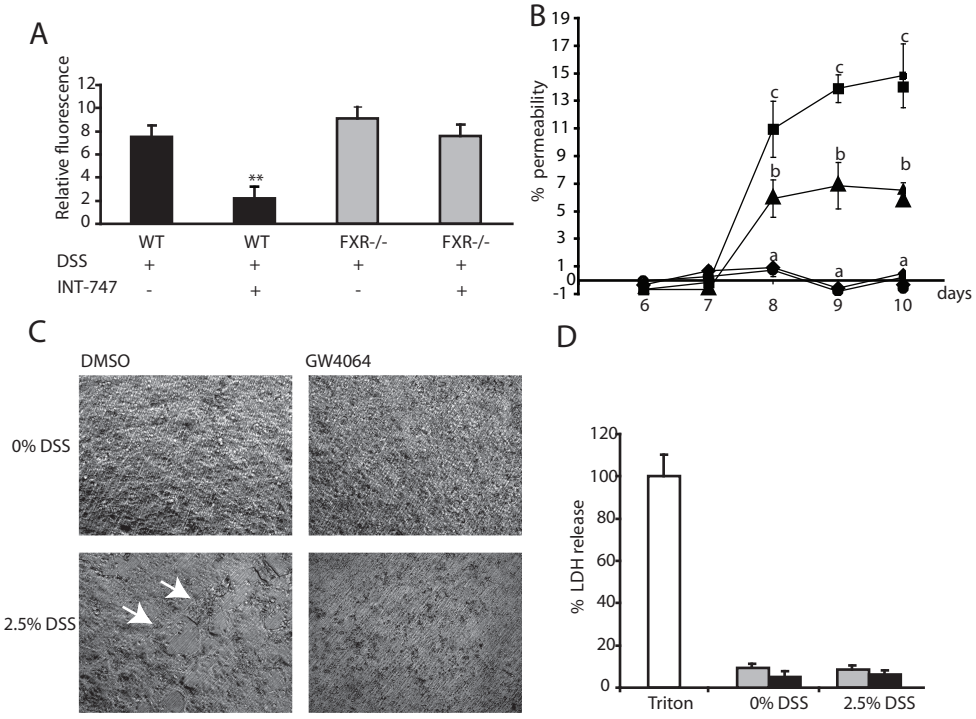

Figure 3: *FXR activation preserves the integrity of the intestinal epithelial barrier in vivo and in vitro.*
(A) *In vivo* intestinal permeability measurement following 7 days of DSS of vehicle- or INT-747-treated WT and Fxr-ko mice. Each data point represents the mean value±SEM. Student's t-test, ** $p<0.01$, compared to WT vehicle-treated mice. (B) Lucifer yellow permeability measured in differentiated Caco-2 cell monolayers (0% DSS ◆; 0% DSS GW4064 ●; 2.5% DSS ■; 2.5% DSS GW4064 ▲); ANOVA statistical analysis with Bonferroni post-hoc test was performed for each time point; significant differences ($p<0.05$) are indicated by different letters. (C) Bright field microscopy pictures and (D) LDH assay after 8 days of DSS treatment. Caco-2 cells incubated with Triton 1% (white bar), DMSO (light grey bar) or GW4064 (black bar).

dramatically at day 8: cells detached from each other and the transwell, leaving gaps in the monolayer (Figure 3C). Medium taken from the same experiment (day 8) did not show increased LDH release in Caco-2 cells upon DSS treatment compared to untreated cells at the same time point (Figure 3D), indicating that the DSS-induced permeability is not caused by cell damage.

Fxr activation inhibits inflammatory gene expression and promotes anti-bacterial gene expression in DSS-induced colitis.

Since IBD in humans is thought to result from both compromised intestinal epithelial barrier function and dysregulation of the mucosal immune system, we investigated whether Fxr modulates inflammatory gene expression in DSS-induced colitis. In WT DSS-treated mice, INT-747 significantly decreased colonic mRNA expression of the pro-inflammatory genes *Il-1β*, *Il-6* and the *macrophage attractant protein-1* (*Mcp-1*) (Figure 4A). These data are consistent with the significantly reduced colonic protein concentrations of Il-6, Mcp-1 and Mpo in INT-747-treated mice (Supplementary Figure 2 E-G). Nitric oxide (NO), angiogenin 1 (Ang1) and cathelicidin are known to

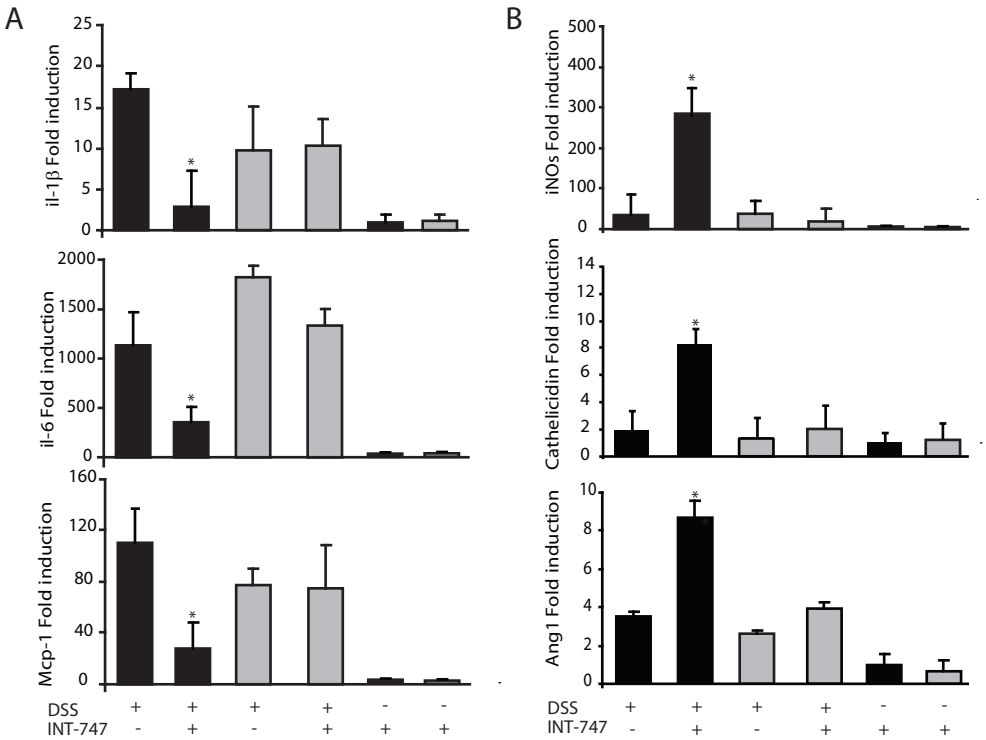

Figure 4: *Fxr activation inhibits inflammatory gene expression and promotes antibacterial gene expression in DSS-induced colitis.*

(A) qRT-PCR of *Il-1β*, *Il-6* and *Mcp-1*, and (B) of *iNOs* and *cathelicidin* (both in the colonic mucosa) and *Ang1* (in the ileal mucosa) of WT (black bars) and Fxr-ko mice (grey bars). Expression was normalized to Gapdh, and each bar represents the mean value±SEM. Student's t-test, *p<0.05 compared to WT DSS vehicle-treated mice.

have microbicidial properties[32-34] and their increased expression may therefore exert positive effects on inflammation status. We show that *inducible Nitric Oxide synthase* (*iNOs*) and *cathelicidin* in the colon and *angiogenin 1* (*Ang1*) expression in the ileum were significantly induced by INT-747 treatment in WT but not Fxr-ko mice (Figure 4B). These data indicate that Fxr activation decreases expression of several inflammatory genes and promotes expression of microbicidial genes most probably contributing to the amelioration of DSS-induced colitis in WT mice.

FXR activation decreases TNBS-dependent intestinal inflammation.
We next verified the ameliorating effects of FXR activation on DSS-induced colitis in another well-known murine IBD model[35]. TNBS was administered to WT mice two times with a 7 day interval. This protocol resulted in severe colitis in WT C57BL/6 mice. Again, daily administration of INT-747 decreased body weight loss (Figure 5A), infiltration of inflammatory cells, degeneration of the epithelial layer and goblet cell loss (Figure 5B), as well as intestinal permeability (Figure 5C). In concordance with these results, mRNA expression of pro-inflammatory cytokines *Il-1β* and *Mcp-1* was decreased while the antimicrobial peptide *iNOs* was significantly induced in INT-747-treated mice (Figure 5D). These results confirm that Fxr activation ameliorates intestinal inflammation in a separate, well-established model of experimental colitis.

FXR activation decreases pro-inflammatory cytokine expression in intestinal cells.
We next explored the possible contribution of enterocytes in improving inflammation status *in vitro*. We used a well-established cellular model for cytokine expression in human enterocytes, HT29 cells[36,37]. As in Caco-2 cells, FXR was expressed exclusively in fully differentiated HT29, and GW4064 induced the FXR target gene *ileal bile acid binding protein* (*IBABP*), indicating that FXR is functionally active (Supplementary Figure 4C and D). As expected, upon TNFα treatment *IL-1β* mRNA expression was dramatically induced compared to control. GW4064 treatment alone did not affect *IL-1β* expression (Figure 6A). Strikingly, the TNFα-mediated induction in *IL-1β* mRNA expression was almost completely abolished upon co-treatment with GW4064, indicating that FXR activation counteracts pro-inflammatory gene expression of IL-1β in intestinal cells. Expression of NF-κB subunits *RelA* and

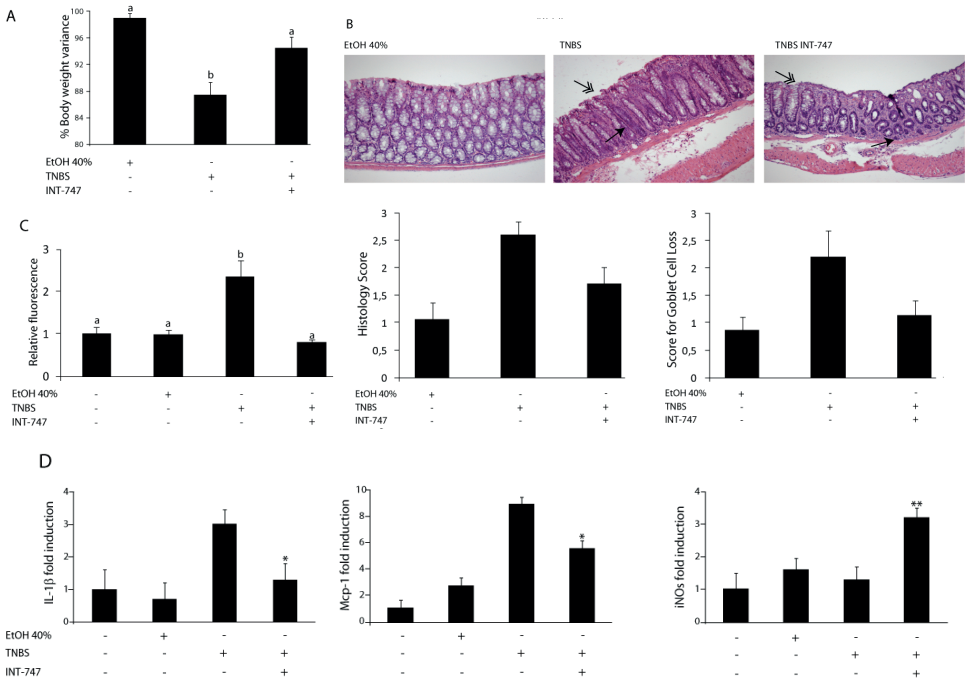

Figure 5: *INT-747-dependent Fxr activation confers protection against TNBS-induced colitis.*

(A) Body weight variance (%) of WT mice treated with or without TNBS, in the presence or absence of INT-747 (5mg/Kg/day/mouse). ANOVA statistical analysis was performed, significant differences ($p<0.05$) are indicated by different letters. (B) Representative hematoxylin and eosin-stained colonic sections, histology and goblet cell loss score; Arrows point at inflammatory infiltrates (→) and epithelial degeneration (⊢→). Magnification 100x. Each bar represents the mean value±SEM. ANOVA statistical analysis with Bonferroni post-hoc test was performed, significant differences ($p<0.05$) are indicated by different letters. (C) *In vivo* intestinal permeability measurements at the day of sacrifice of untreated, controls (EtOH40%) and TNBS of vehicle- or INT-747-treated mice. Each data point represents the mean value± EM. ANOVA statistical analysis with Bonferroni post-hoc test was performed, significant differences ($p<0.05$) are indicated by different letters. (D) qRT-PCR of *Il-1β*, *Mcp-1* and *iNOs* in the mouse colonic mucosa. Expression was normalized to *Gapdh*, and each bar represents the mean value±SEM. Student's t-test, *$p<0.05$, **$p<0.01$ compared to TNBS-vehicle-treated mice.

p50 (Figure 6A) was not decreased upon TNFα and GW4064 co-treatment. Therefore, in order to investigate if FXR decreases *IL-1β* mRNA expression by inhibiting the NF-κB transcriptional activity, HEK293 cells were transfected with a κB reporter plasmid alone or in combination with FXR and RXR, and

incubated with TNFα, GW4064 or both (Figure 6B). In cells transfected with the κB-reporter alone, TNFα increased NF-κB activity. GW4064 had no effect on basal luciferase expression and on TNFα-mediated NF-κB activity. In cells co-transfected with FXR, GW4064 almost completely abolished the TNFα-induced κB-responsive luciferase expression. An FXR mutant defective in ligand binding (W469A) did not repress NF-κB activity, indicating that ligand-activated FXR inhibits NF-κB transcriptional activity *in vitro*.

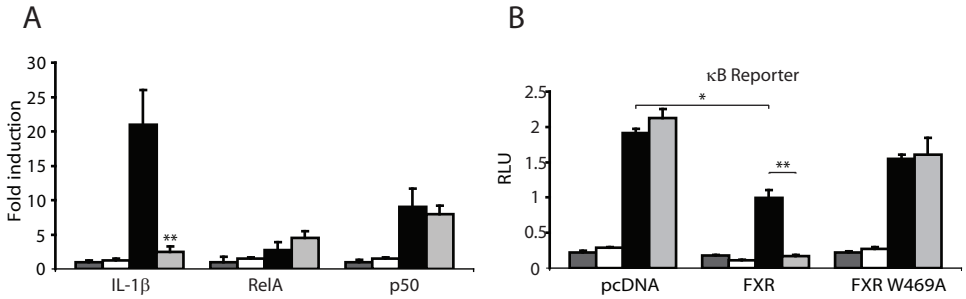

Figure 6: *FXR activation decreases pro-inflammatory cytokine expression in intestinal cells.* (A) qRT-PCR analysis of *IL-1β*, *RelA* and *p50* expression in differentiated HT29 cells incubated with DMSO (dark grey bars), GW4064 (1μM, 24h, white bars) and TNFα (500U/mL, 6h, black bars) or both (light grey bars). (B) Reporter assays of NF-κB transcriptional activity. Cells were treated with DMSO (dark grey bars), GW4064 (1μM, white bars) and/or TNFα (500U/mL, black and light grey bars, respectively) for 24h. Each bar represents the mean value±SD; Student's t-test, *p<0.05, **p<0.01 compared to TNFα treated cells. Data are from a representative experiment out of 4 performed.

FXR activation decreases TNFα production by human immune cells.

As TNFα is a clinically validated target in IBD, we have examined the capacity of INT-747 to inhibit TNFα secretion by different human immune cell types: PBMCs, monocytes, and dendritic cells. INT-747 inhibited dose-dependent TNFα secretion by PBMCs, whereas no inhibition was induced by Cholic Acid (CA) and only a marginal one by high concentrations of Chenodeoxycholic Acid (CDCA) (Figure 7A). Similar results were obtained with CD14+ monocytes (Figure 7B). INT-747 also inhibited the GM-CSF/IL-4-induced differentiation of CD14+ monocytes into dendritic cells (Figure 7C). Dendritic cells differentiated in the presence of INT-747 showed a markedly reduced capacity to secrete TNFα in response to LPS stimulation (Figure 7D). Thus, *in vitro* addition of INT-747 inhibits TNFα secretion by different human and

mouse immune cell types, in particular professional antigen-presenting cells. In line with these results, *in vivo* treatment with INT-747 also significantly decreased serum TNFα and IFNγ levels following LPS-induced systemic inflammatory response in phagocytic mononuclear cells obtained from mouse peritoneal exudate cells (PECs), without affecting their recruitment (Supplementary Figure 5). Together these results indicate that *in vitro* and *in vivo* FXR activation inhibits inflammatory responses in various isolated human immune cells.

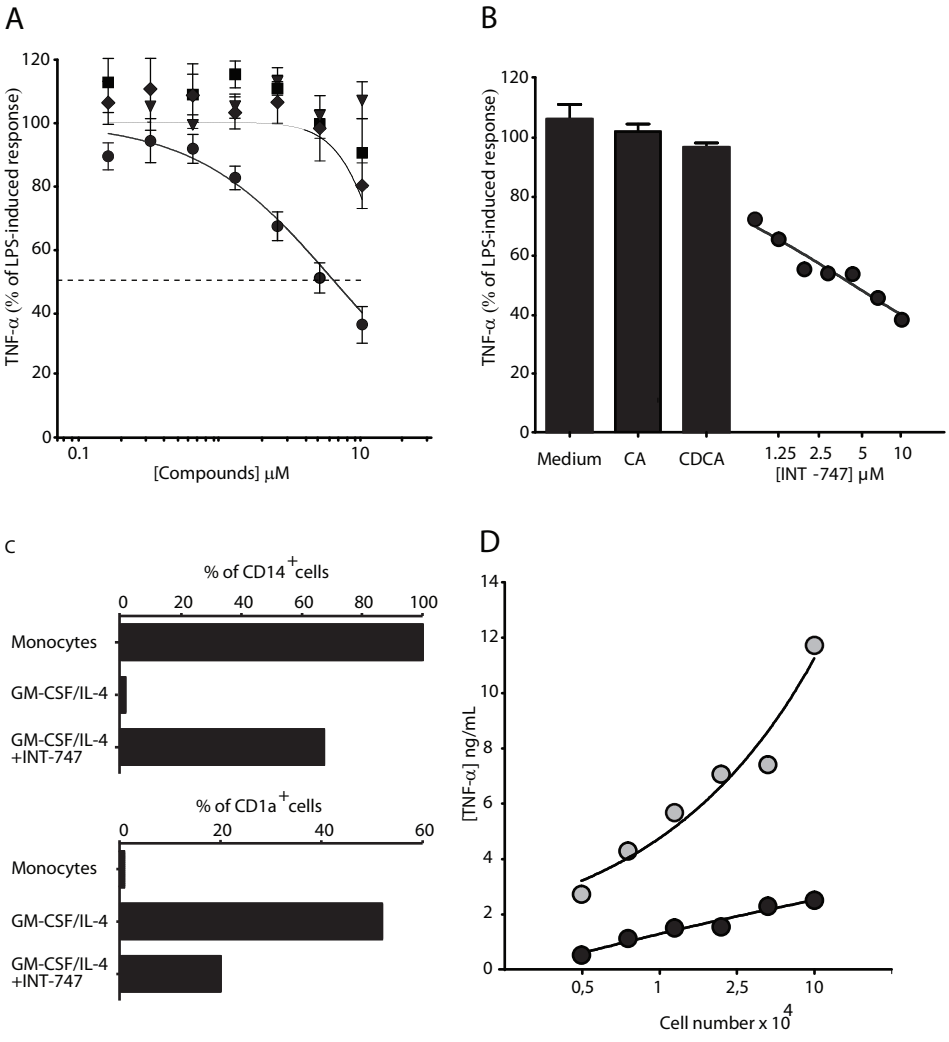

Figure 7: *INT-747-dependent FXR activation decreases TNFα production by human inflammatory cells.*
(A) TNFα secretion by LPS-stimulated PBMCs incubated with the indicated concentrations of Cholic Acid (CA), Chenodeoxycholic Acid (CDCA), or INT-747 (medium ▼; CA ■; CDCA ◆; INT-747 ●). (B) TNFα secretion by LPS-stimulated CD14+ monocytes incubated for 24h with 10μM CA or CDCA, or with the indicated concentrations of INT-747. Values are expressed as percentage of control LPS-induced response (PBMCs, 1059±91; monocytes, 1737±80 pg/ml) and represent the mean±SEM of at least 3 experiments. (C) Monocyte-derived dendritic cells generated after 5 days culture with GM-CSF and IL-4 in the presence or absence of 10 μM INT-747 were double stained with anti-CD14 and anti-CD1a mAbs and analyzed by flow cytometry. (D) The same cells were stimulated with LPS for 24h and TNFα production was determined (GM-CSF/IL4 vehicle, light grey circles; GM-CSF/IL4 INT-747, black circles). Data are from a representative experiment out of 3 performed.

INT-747-induced FXR activation of ex-vivo lamina propria mononuclear cells from IBD patients reduces inflammatory cytokine secretion.
As shown above, INT-747 is a potent inhibitor of pro-inflammatory cytokines *in vitro* in human immune cells. To assess the capacity of this compound

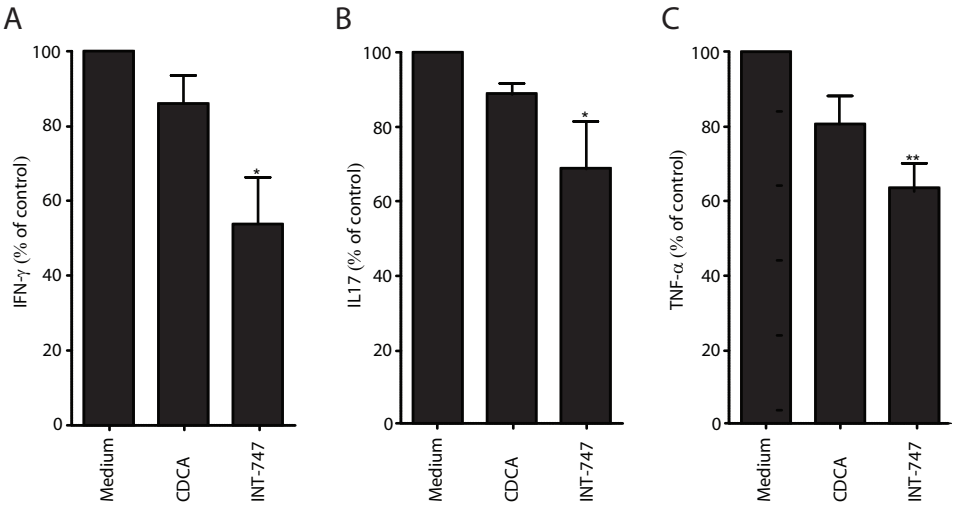

Figure 8: *INT-747 inhibits IFNγ, IL-17 and TNFα production by lymphocyte-enriched LPMCs from IBD patients.*
LPMCs from 4 IBD patients (2 CD and 2 UC) were stimulated for 24-72h with αCD2 and αCD28. (A) IFNγ (72h), (B) IL-17 (24h) and (C) TNFα (24h) secretion was determined by ELISA. Values are expressed as percentage of control responses (IFNγ, 1754±1027; IL-17, 2029±1722; TNFα, 341±118 pg/ml). Student's t-test, *p<0.05, **p< 0.01, compared to medium.

to inhibit key components of the inflammatory response in the human intestine, we have examined its capacity to inhibit pro-inflammatory cytokines produced by activation of resident cells from the inflamed target organ, the lamina propria mononuclear cells (LPMCs) from IBD tissue. Lymphocytes purified from LPMCs were activated with a cocktail of antibodies targeting CD2 and CD28. INT-747 significantly inhibits IFNγ, IL-17 and TNFα production by LPMCs (Figure 8A-C). These results indicate that anti-inflammatory properties of INT-747 observed in PBMCs, monocytes, and dendritic cells from healthy volunteers also occur in LPMCs of IBD patients.

DISCUSSION

Current medical options for patients with IBD, including glucocorticoids, 5-ASA and anti-TNFα antibody treatment, reduce the pro-inflammatory response. Although they have all been shown to interfere with NF-kB signalling *in vitro*, their mechanism of action in IBD is largely elusive and may depend on mechanisms other than NF-kB inhibition. In addition, these treatments are often accompanied by side effects (sepsis, opportunistic infections, TBC, lymphoma, diabetes, osteoporosis) and significant numbers of treatment failures, highlighting a need for alternative strategies in the treatment of IBD. Here we describe pharmaceutical activation of FXR as a potential new treatment option for IBD.
In this study, we have used two independent murine IBD models: DSS- and TNBS-induced colitis. Although there are differences with human IBD, these models share many clinical and pathological features with human IBD with regard to loss of barrier function and inflammatory response. INT-747, a potent and selective FXR agonist[38], was efficiently taken up in the enterohepatic circulation and strongly activated Fxr in the intestine, as evidenced by markedly induction of Fxr target gene expression. Our findings revealed that INT-747 significantly decreased the severity of DSS- and TNBS-induced colitis in mice, as indicated by decreased body weight loss, colon shortening, and rectal bleeding as well as improvement of colonic histology. The improvement in DSS-induced IBD was not seen in Fxr-ko mice, demonstrating that the amelioration of colitis by INT-747 requires Fxr. Fxr-ko mice do not appear to be more susceptible to DSS colitis than WT mice and this

might be due to the duration of the DSS protocol, the young age of the mice[11] and the micro-environmental conditions. Nevertheless, the basal intestinal permeability of Fxr-ko mice is two times higher compared to WT mice (Supplementary Figure 3). This is in line with the higher bacterial translocation observed in Fxr-ko mice[15]. Since IBD is thought to result from both dysregulation of the mucosal immune system and compromised intestinal epithelial barrier function in genetically predisposed individuals[2,39], we explored the ability of FXR to counter-regulate intestinal inflammation at different levels. Enterocytes are known to serve as immuno-effector cells and are capable of secreting cytokines and chemokines to promote inflammation[40]. This results in infiltration of macrophages and other immune cells to the site of intestinal inflammation. FXR activation abrogates the expression of the pro-inflammatory gene *IL-1β* in human enterocyte-like HT29 cells *in vitro*, and this finding is in line with the capacity of Fxr activation to nearly normalize DSS- and TNBS-induced colonic pro-inflammatory gene expression and to markedly reduce inflammatory infiltrates. In addition, we show that expression of several antibacterial defence gene was significantly induced by INT-747 treatment in WT mice (Figure 4B and 5D), suggesting that Fxr activation may contribute to control bacterial overgrowth. The composition of enteric microflora is linked to the initiation and progression of intestinal inflammation, and the intriguing possibility that FXR plays a role in the modification of the intestinal ecology should be subject of future studies as a putative possibility for both pathogenesis and treatment of IBD. In addition, we observed that INT-747 overcomes DSS- and TNBS-induced increased intestinal permeability in WT mice, in concurrence with decreased endothelial ulceration in colons of these mice. Several cytokines are known to increase permeability in intestinal epithelial monolayer (e.g. TNFα, IFNγ, IL-4 and IL-13)[41,42], by modulating tight junction protein expression and localization. The observed inhibition of cytokine expression by Fxr activation may therefore represent a possible mechanism preserving intestinal permeability. Alternatively, Fxr may have a direct effect on the integrity of the intestinal mucosa. This hypothesis is supported by *in vitro* studies showing that FXR activation decreased DSS-induced detachment of human enterocyte-like Caco-2 cells from the monolayer. At this stage, the precise mechanisms by which Fxr improves barrier integrity are still unclear. Besides aberrant immune response and loss of intestinal barrier integrity, IBD

patients display various degrees of goblet cell loss[8]. Goblet cells are intestinal mucin-secreting cells forming the mucus layer that protects the mucosal surface from antigens and thus maintains intestinal barrier function. Also in our experimental colitis models significant goblet cell loss was detected which was partially prevented by INT-747, suggesting that Fxr activation may ameliorate intestinal barrier function also at the level of goblet cell counts. In agreement with Vavassori *et al.*,[11] we show that INT-747 dependent FXR activation inhibits the inflammatory response in immune cells. However, we extend their results obtained in mice and human immortalized immune cell lines to various primary human immune cell types (PBMCs, CD14+ monocytes and monocyte-derived dendritic cells). Moreover, we show that the anti-inflammatory properties of FXR activation also reduce inflammatory signalling in lamina propria mononuclear cells (LPMCs) isolated from IBD patients. In addition, FXR activation prevented the *in vitro* differentiation of dendritic cells. These results are in line with the marked reduction in inflammatory infiltrates in our models of murine colitis. In conclusion, our results indicate that FXR is an important player in the counter regulation of intestinal inflammation. Although the exact mechanism is still unclear, it is very likely that the protection against experimental colitis may not rely on a single FXR-dependent mechanism. The multi-level protection against intestinal inflammation provides a clear rationale to further explore FXR agonists as a novel therapeutic strategy for IBD. Currently, Phase 1 and 2 studies with INT-747 are being performed in patients with metabolic and chronic liver diseases, indicating the safe applicability of this class of compounds for the treatment of human disease[43].

Acknowledgement
We thank Lorena Salvatore for expert histology and microscopy assistance. Martin de Smet is acknowledged for his assistance with the biliary bile salt measurements.

Competing interests
GP, GL and LA are with Intercept Pharmaceuticals. The other authors declared no competing interests.

Funding
LWJK is funded by the by the University Utrecht High Potentials program, AM by the Italian Association for Cancer Research (AIRC, Milan, Italy), European

Research Council Starting Independent Grant Ideas, Italian Ministry of Health and Education (FIRB- IDEAS 2008 RBID08C9N7), European Community's Seventh Framework Programme FP7/2007– 2013 under Grant Agreement No. 202272 (LipidomicNet), Cariplo Fundation Milan, Telethon Fundation (GGP08259), University of Bari. SM is a fellow of CariSPAQ (L'Aquila, Italy). SWCVM is funded by the Netherlands Organisation for Scientific Research (NWO).

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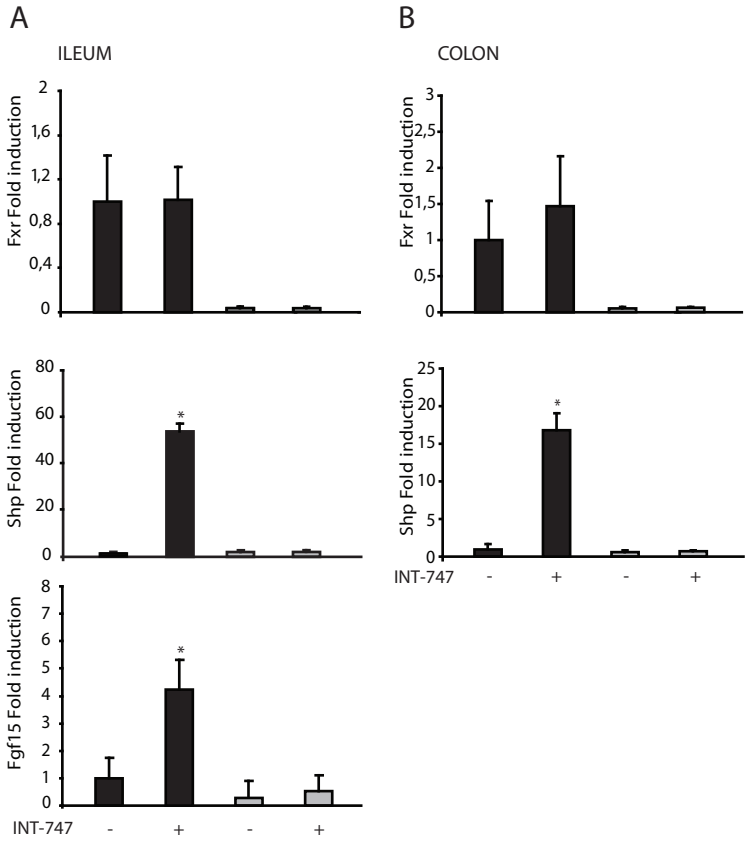
SUPPLEMENTARY FIGURES.



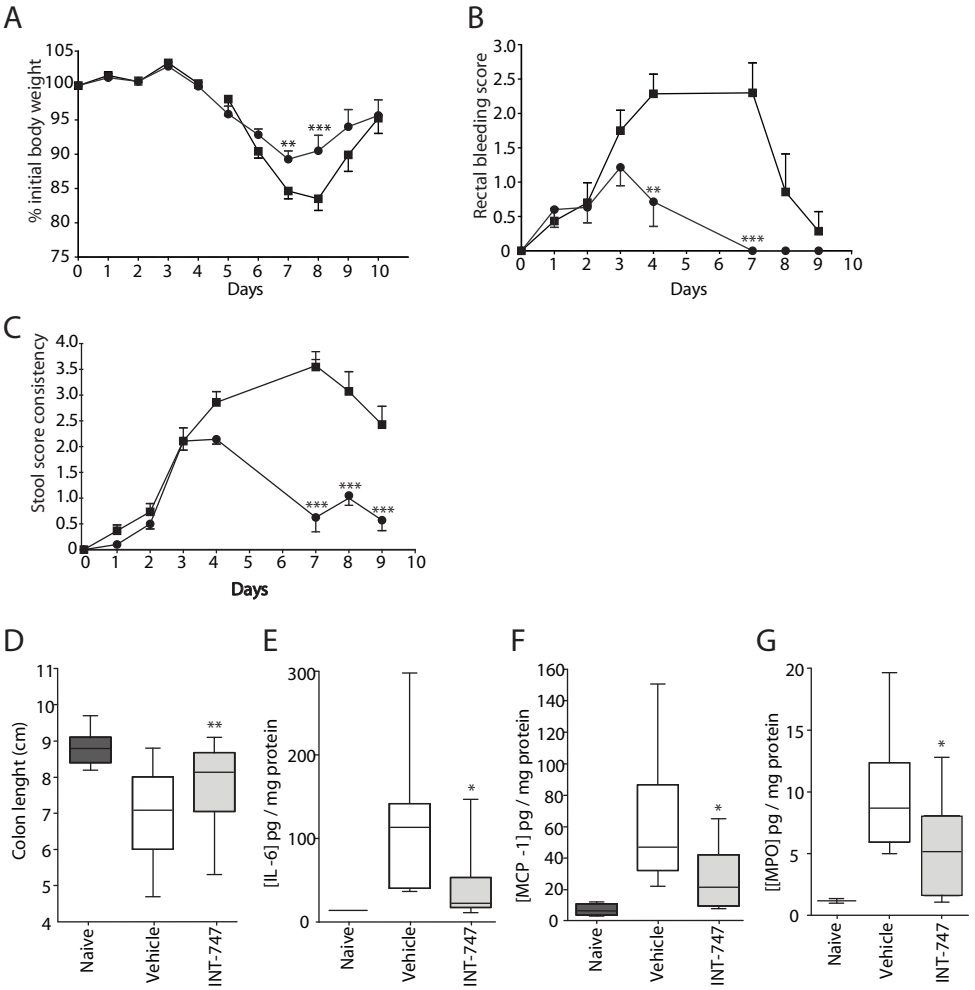
Supplementary Figure 1: *Fxr is expressed and activated by INT-747 in ileum and colon of WT mice.*

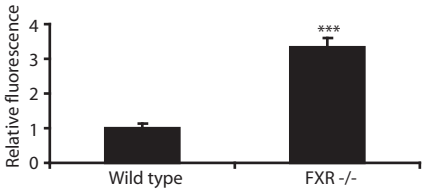
qRT-PCR analysis of (A) *Fxr*, *Shp* and *Fgf15* in ileum and (B) *Fxr* and *Shp* in colon of WT (black bars) and Fxr-ko mice (grey bars). Each bar represents the mean value±SEM. Student's t-test, *p<0.05 compared to WT vehicle-treated mice.

Supplementary Figure 2: *INT-747 ameliorates clinical symptoms and inhibits secretion of inflammatory mediators in DSS-induced murine colitis.*

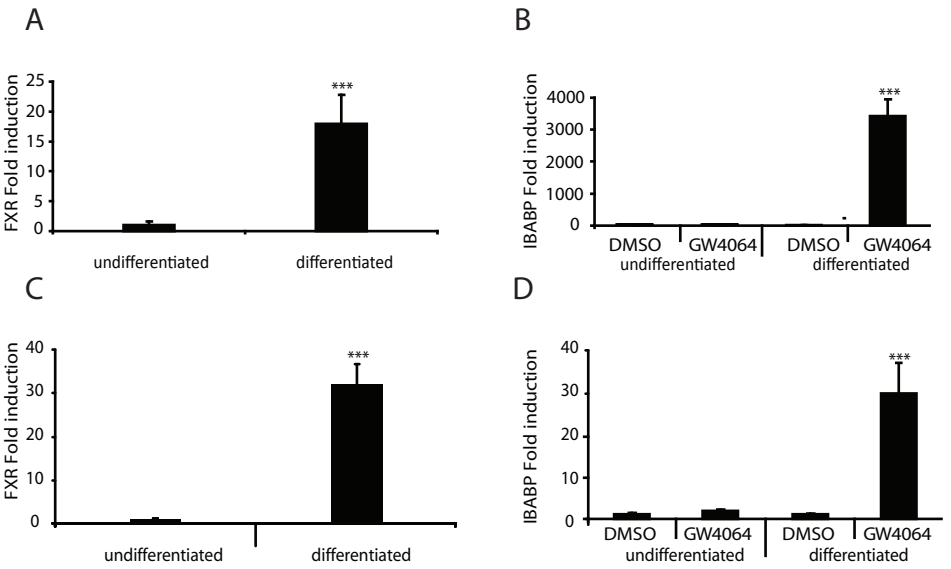
Colitis was induced by treatment with 3% DSS for 5 days, and then mice were resumed on water for the remainder of the experiment. From day 0 to day 5 mice were treated orally with 10mg/kg of INT-747 or vehicle. Mice were checked daily for colitis symptoms, namely (A) percentage of initial body weight, (B) rectal bleeding score, (C) stool consistency (vehicle ■; INT-747 ●), and (D) colon length. Homogenates for analysis of cytokine concentrations were prepared from colon samples using a tissue homogenizer. (E) levels of Il-6, (F) Mcp-1, and (G) Mpo were determined in colon homogenates. Each point in panels A, B and C represents the mean ± SEM of 20-30 mice/group. ANOVA

statistical analysis was performed, *p<0.05; **p<0.01; ***p<0.001, compared to vehicle-treated mice.
Each point in panels D, E, F and G represents the mean ± SEM of 10-20 mice/group. Student's t test, *p<0.05, **p<0.01 compared to vehicle treated mice.

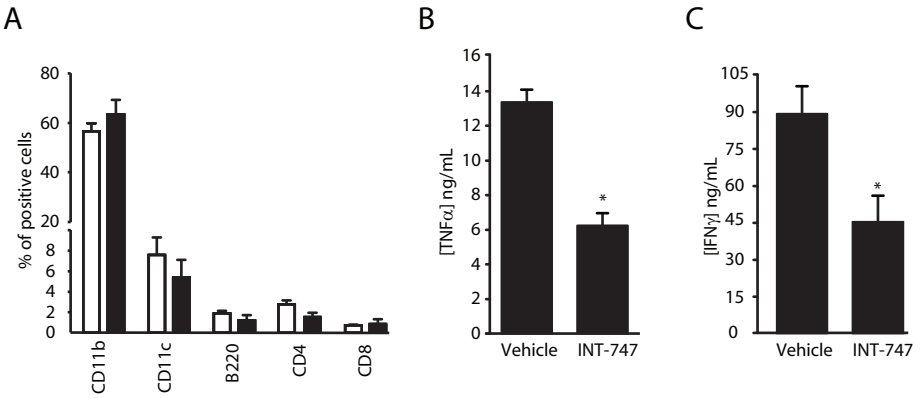




Supplementary Figure 3: *Fxr-ko mice have impaired intestinal barrier.*
In vivo intestinal permeability measurement in basal condition of WT and Fxr-ko mice. Each bar represents the mean value±SEM. Student's t-test, ***p<0.001compared to WT mice.



Supplementary Figure 4: *FXR is expressed and functionally active in differentiated enterocyte-like Caco-2 and HT29 cells.*
qRT-PCR analysis of *FXR* and *IBABP* expression in undifferentiated versus differentiated Caco-2 (A, B) and HT-29 (C, D) cells incubated for 24h with DMSO or GW4064 (1µM). Each bar represents the mean value±SEM; Student's t-test, ***p<0.001 compared to undifferentiated cells. Data are from a representative experiment out of 3 performed.



Supplementary Figure 5: *INT-747 inhibits inflammatory responses in vivo.*
(A) Cytofluorimetric analysis of thioglycollate-elicited peritoneal exudate cells (PECs). PECs were elicited by administration of 3% thioglicollate solution in 8week-old female C57BL/6 mice and then stimulated with 100ng/ml LPS for 24h, after plastic adherence or immediately stained with anti-mouse monoclonal antibodies, anti-CD11b, anti-CD11c, anti-CD45R/B220, anti-CD4 and anti-CD8. The composition of PECs is not affected by INT-747 treatment.
Systemic inflammatory response was induced by intraperitoneal injection of 10 mg/kg LPS. INT-747 was administered orally at 24h and 1h before LPS injection. After 6h, blood was taken and serum was stored at -80°C until TNFα and IFNγ determination. Serum TNFα (B) and IFNγ production by LPS-stimulated thioglycollate-elicited PECs (C) is significantly reduced after in vivo pre-treatment with 10mg/kg/mouse INT-747.
Student's t-test, *p <0.05 compared to vehicle.

SUPPLEMENTARY TABLE 1: *qRT-PCR Primers (5'-3')*

Mouse

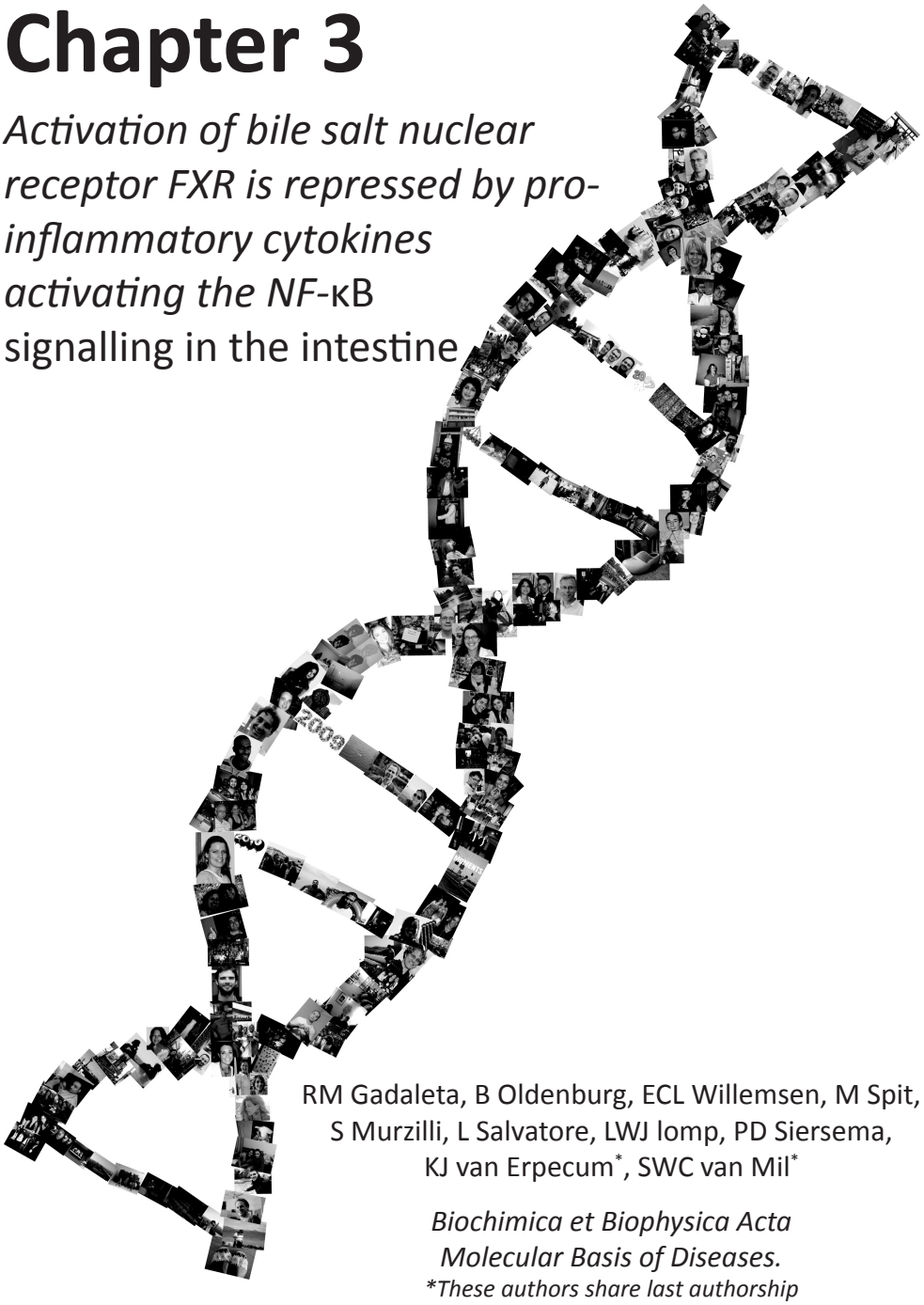
Gapdh F	GCAAAGTGGAGATTGTTGCCAT
Gapdh R	CCTTGACTGTGCCGTTGAATTT
Fxr F	TGAGAACCCACAGCATTTCG
Fxr R	GCGTGGTGATGGTTGAATGTC
Fgf15 F	AAAACGAACGAAATTTGTTGGAA
Fgf15 R	ACGTCCTTGATGGCAATCG
Shp F	CGATCCTCTTCAACCCAGATG
Shp R	AGGGCTCCAAGACTTCACACA
Il-1β F	CCTCAATGGACAGAATATCAACCAA
Il-1β R	TCTCCTTGTACAAAGCTCATGGAG
Mcp-1 F	CATCCACGTGTTGGCTCA
Mcp-1 R	GATCATCTTGCTGGTGAATGAGT
Il-6 F	GCTACCAAACTGGATATAATCAGGA
Il-6 R	CCAGGTAGCTATGGTACTCCAGAA
iNOs F	CAGGAGGAGAGAGATCCGATTTA
iNOs R	GCATTAGCATGGAAGCAAAGA
Ang1 F	AGCGAATGGAAGCCCTTACA
Ang1 R	CTCATCGAAGTGGACCGGCA
Cathelicidin F	GCCGCTGATTCTTTTGACAT
Cathelicidin R	AATCTTCTCCCCACCTTTGC

Human

GAPDH F	TGTTGCCATCAATGACCCCTT
GAPDH R	CTCCACGACGTACTCAGCG
FXR F	CTACCAGGATTTCAGACTTTGGAC
FXR R	GAACATAGCTTCAACCGCAGAC
SHP F	AGGGACCATCCTCTTCAACC
SHP R	TTCACACAGCACCCAGTGAG
IBABP F	TCAGAGATCGTGGGTGACAA
IBABP R	TCACGCGCTCATAGGTCA
IL-1β F	CTCGCCAGTGAAATGATGGCT
IL-1β R	GTCGGAGATTCGTAGCTGGAT
RelA F	CGGGATGGCTTCTATGAGG
RelA R	CTCCAGGTCCCGCTTCTT
p50 F	AGAAGTCTTACCCTCAGGTCAAA
p50 R	TCCAGCAGTTACAGTGCAGAT

Chapter 3

Activation of bile salt nuclear receptor FXR is repressed by pro-inflammatory cytokines activating the NF-κB signalling in the intestine



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Biochimica et Biophysica Acta Molecular Basis of Diseases.

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ABSTRACT

Background & aims: Hyperactivation of NF-κB is a key factor in the pathophysiology of inflammatory bowel disease (IBD). We previously showed that the bile salt nuclear Farnesoid X Receptor (FXR) counter-regulates intestinal inflammation, possibly via repression of NF-κB. Here, we examine whether mutual antagonism between NF-κB and FXR exists. ***Methods:*** *FXR* and its target genes *IBABP* and *FGF15/19* expression were determined in HT29 colon carcinoma cells and *ex vivo* in intestinal specimens of wild type (WT) and Fxr-ko mice, treated with/without FXR ligands (GW4064/INT-747) and inflammatory stimuli (TNFα/IL-1β). In addition, FXR activation was studied *in vivo* in WT and Fxr-ko mice with DSS-colitis. The involvement of NF-κB in decreasing FXR activity was investigated by reporter assays and Glutathione S-transferase pulldown assays. ***Results:*** FXR target gene expression was highly reduced by inflammatory stimuli in all model systems, while *FXR* mRNA expression was unaffected. In line with these results, reporter assays showed reduced FXR transcriptional activity upon TNFα/IL-1β stimulation. We show that this reduction in FXR activity is probably mediated by NF-κB, since overexpression of NF-κB subunits p50 and/or p65 also leads to inhibition of FXR activity. Finally, we report that p65 and p50 physically interact with FXR *in vitro*. ***Conclusions:*** Together, these results indicate that intestinal inflammation strongly reduces FXR activation, probably via NF-κB-dependent tethering of FXR. Therefore, FXR not only inhibits inflammation, but also is targeted by the inflammatory response itself. This could result in a vicious cycle where reduced FXR activity results in less repression of inflammation, contributing to development of chronic intestinal inflammation.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation, with potentially severe complications and even mortality[1]. Although the exact aetiology is unclear, it is thought to result from a combination of dysregulation of the mucosal immune system, hyper-reactive against the intestinal microbiota, and compromised intestinal epithelial barrier function in genetically predisposed individuals[2]. In IBD patients, the nuclear transcription Factor κappa B (NF-κB) was identified as a key factor in the pro-inflammatory response, resulting in strongly enhanced expression of pro-inflammatory genes and recruitment of excess inflammatory cells to the intestinal wall[3]. The NF-κB complex is a heterodimeric protein predominantly consisting of p65 and p50 sub-units[4]. In the absence of activating signals, the inhibitor protein IκBα retains NF-κB in the cytosol. Presence of pro-inflammatory cytokines (e.g. TNFα, Il-1β), reactive oxygen species or viral products, initiates phosphorylation and subsequent degradation of IκBα, allowing NF-κB to translocate to the nucleus and directly regulate the expression of specific target genes[5]. The bile salt nuclear Farnesoid X Receptor (FXR) is a member of the superfamily of nuclear receptors. Nuclear receptors are ligand-activated transcription factors that, in response to lipophilic ligands (e.g. hormones, vitamins and dietary lipids), regulate many aspects of mammalian physiology, including development, reproduction and metabolism[6,7]. FXR is mainly expressed in the ileum and liver. Once activated by bile salts, it regulates transcription of genes involved in bile salt synthesis, transport and metabolism[8]. FXR binds as a heterodimer with Retinoid X Receptor (RXR) to the FXR responsive elements on the promoters of target genes, such as the small heterodimer partner (SHP), intestinal bile acid binding protein (IBABP) and fibroblast growth factor 15/19 (FGF15/19 in mouse and human, respectively), involved in bile salt homeostasis. We have recently found that pharmacological FXR activation decreases the severity of inflammation and preserves the intestinal barrier integrity in two well-established murine colitis models[9]. In recent years, reciprocal repression between several nuclear receptors (NR) and inflammatory pathways has been described. Activation of several NRs (e.g. Glucocorticoid Receptor, Androgen Receptor and Estrogen Receptor) inhibits inflammation, whereas activation of these nuclear receptors is highly reduced in inflamed tissues, suggesting that repression of nuclear

receptors might be a mechanism required for inflammation to progress[10]. Also, FXR-mediated gene expression was shown to be suppressed during hepatic inflammation[11] and *FXR* mRNA expression was shown to be reduced in inflamed colonic mucosa in a small cohort of Crohn's patients[12]. In the current work, we provide *in vitro*, *ex vivo* and *in vivo* evidence that the inflammatory response reciprocally inhibits activation of FXR and its target genes in the intestine. These complementary findings may have important implications for intestinal inflammation and regulation of bile salt homeostasis in patients with inflammatory bowel disease.

METHODS

Cell culture.

Human embryonic kidney cells HEK293T and human colon carcinoma HT29 (passage number 10-20) cells were grown at 37°C with 5% CO2 in Dulbecco Modified Eagle's Medium (DMEM) GlutaMax™ (4.5 g/L D-Glucose and Pyruvate; Gibco BRL, Breda, the Netherlands), supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Gibco BRL). HT29 cells were maintained in culture for 7 days in order to allow differentiation into mature enterocyte-like cells. Both HT29 and HEK293 cells were treated with DMSO, the synthetic FXR ligand GW4064 (1uM for 24 hours), Tumor Necrosis Factor α (TNFα; 500U/mL, for 1, 6 or 24 hours, as indicated) or Interleukin 1β (IL-1β; 20ng/mL for 24 hours). GW4064 is a synthetic FXR agonist which binds to FXR with an EC 50 of 15 nM[13].

Reporter assays.

HEK293T cells were grown in 96-multiwell plates and co-transfected with empty pGL3, pGL3-IBABP or pGL3-SHP reporters, tK-Renilla and either empty pcDNA or pcDNA-hFXRα2 together with pcDNA-RXRα using the standard calcium phosphate method, as described elsewhere.[14] After 24 hours, cells were incubated with vehicle (DMSO) or 1 μM GW4064 for 24 hours, in presence or absence of either TNFα (500U/ml) or IL-1β (20ng/mL). Alternatively, cells were co-transfected with NF-κB sub-units pcDNA-p50 and/or pcDNA-p65 (kind gift by Dr. E. Kalkhoven, Utrecht, The Netherlands). Cells were lysed and Firefly and Renilla luciferase activity

were measured according to manufacturer's instructions (Promega Dual-Luciferase Reporter Assay System, Promega, Madison, Wisconsin, USA) with the Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium).

Glutathione S-Transferase (GST) Pull-down assay.
GST-pull down experiments were performed as described elsewhere[15]. Briefly, p50 and p65 or FXR were transcribed and translated *in vitro* in the presence of [35S] methionine and incubated with GST and either GST-hFXRα2 or GST-p65 fusion proteins, respectively. Samples were subsequently washed and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Coomassie brilliant blue was used to visulize GST proteins. [35S]-labelled proteins were detected by autoradiography and analyzed with Storm 820 apparatus (Molecular dynamics, Pharmacia Biotechnology, Amersham Biosciences, Diegem, Belgium).

Animals.
Nine-to-twelve weeks old male wild type (WT) C57BL/6J mice were obtained from Charles River Laboratories. Pure strain male C57BL/6J Fxr-ko mice (Dr. D.J. Mangelsdorf, Southwestern Medical Center, Dallas, TX) were kindly provided by Dr. Frank Gonzalez (NIH, Bethesda, MD). Mice were fed ad libitum and housed in a temperature- and light-controlled room. All experiments were approved by the Ethical Committee of the Consorzio Mario Negri Sud and by the Italian Ministry of Health.

Ex-vivo culturing of murine ileal specimens.
Ileal samples of C57Bl/6 WT (n=4) and Fxr-ko (n=4) mice were collected, washed with PBS, divided into 6 pieces of approximately equal size and kept in culture for 24 hours at 37°C with 5% CO2 in Dulbecco Modified Eagle's Medium (DMEM) GlutaMax™ (4.5 g/L D-Glucose and Pyruvate; Gibco BRL, Breda, the Netherlands), supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Gibco BRL). The specimens were treated with DMSO, GW4064 (1μM) and/or either TNFα (500U/mL) or IL-1β (20ng/mL). After 24 hours mRNA was isolated (see below).

FXR activation and induction of murine colitis.
Pharmacological activation of Fxr in mice was accomplished by daily

oral gavage with 5mg/kg/day INT-747 (6-ethyl-chenodeoxycholic acid, Obeticholic acid, Intercept Pharmaceuticals Inc, New York, NY)[16] or vehicle (1% wt/vol methyl-cellulose) from 3 days prior to induction of colitis, and continued until the end of the experiments. INT 747 is a semi-synthetic FXR ligand activating the receptor with an EC 50 of approximately 100 nM[16]. Colitis was induced by administration of 2.5% (wt/vol) Dextran Sodium Sulphate (DSS; MW 36-50 kDa, MP Biochemicals Inc., The Netherlands) in drinking water for 10 days (n=8-10 mice per group)[17].

mRNA extraction and qRT-PCR analysis.
Ileal specimens were homogenized (Omni TH tissue homogenizer, Omni International, Kennesaw, USA) and RNA was isolated using RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The quantity, quality and integrity of isolated mRNA were confirmed by absorption measurement and RNA gel electrophoresis. Subsequently cDNA was generated from 500 ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Roche, Basel, Switzerland). qRT-PCR analysis was carried out using SYBR green PCR master mix (Biorad, Veenendaal, The Netherlands) and a MyIQ real time PCR cycler (Biorad). Values were quantified using the comparative threshold cycle method and *FXR* and target gene mRNA expression was normalized to *GAPDH*. Primers are listed in Supplementary Table 1.

Statistical analysis.
Results are expressed as means ±SEM or ±SD as indicated in the figure legends. ANOVA or Student's t test was performed with GraphPad Software (GraphPad Software, Inc., Avenida de la Playa, La Jolla, USA), as appropriate. Two-sided p-values <0.05 are considered statistically significant and are expressed as *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

RESULTS

TNFα inhibits FXR target gene expression in differentiated HT29 cells.
To study the effects of an inflammatory stimulus on FXR target gene expression, we treated undifferentiated and differentiated HT29 colon

carcinoma cells with TNFα in presence or absence of the FXR synthetic ligand GW4064. In undifferentiated enterocytes, none of the treatments exerted any effects on *FXR*, *IBABP* or *SHP* expression. *FXR* is expressed and functional exclusively in fully differentiated HT29 cells (Figure 1A), in line with its *in vivo* intestinal expression, which is limited to the villi of the enterocyte[18]. There was no significant difference in *FXR* mRNA expression between the various experimental conditions in differentiated cells (Figure 1A). GW4064 markedly induced the FXR target genes *IBABP* and *FGF19*, only in differentiated cells (Figure 1B and C). Co-treatment with TNFα dramatically reduced *IBABP* and *FGF19* expression in a time-dependent manner, indicating that in the presence of a pro-inflammatory stimulus, FXR activation is inhibited (Figure 1B and C). TNFα treatment alone did not affect *IBABP* and *FGF19* mRNA expression (Figure 1B and C).

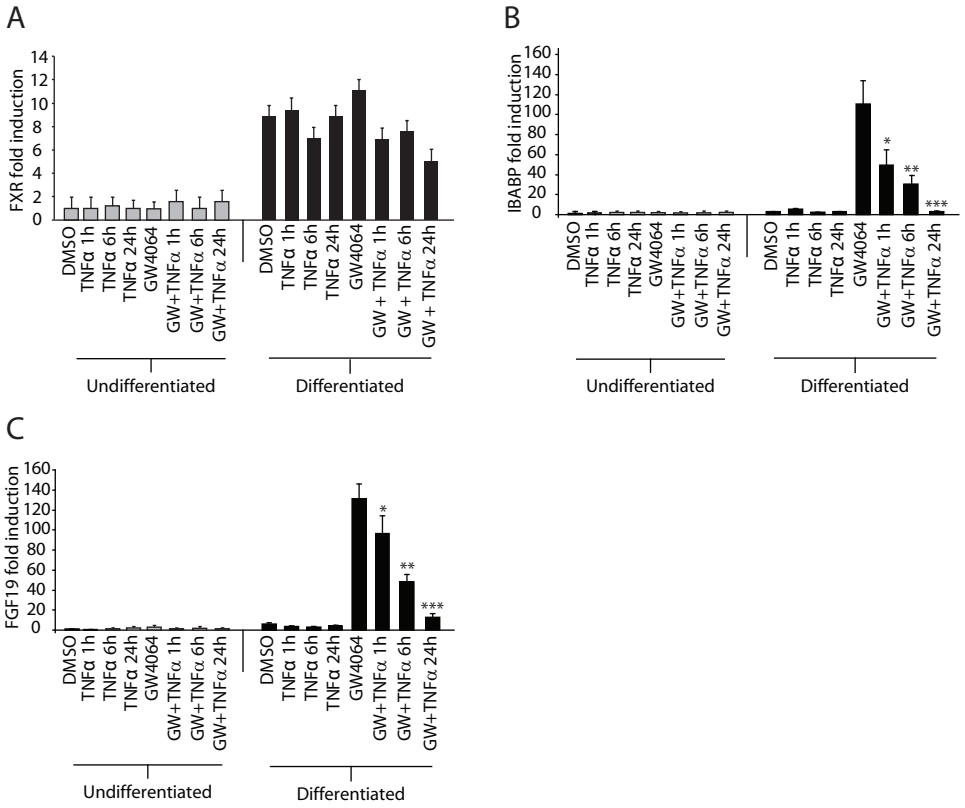

Figure 1: *TNFα decreases Fxr target gene expression in colon carcinoma-derived HT29 cells.* HT29 cells were differentiated by culturing them 7 days post-confluency (black bars).

Undifferentiated cells served as control (grey bars). mRNA expression of *FXR* (A), *IBABP* (B) and *FGF19* (C) in HT29 cells incubated with DMSO, the FXR agonist GW4064 (1μM, 24h), TNFα (500U/mL, 1h, 6h, 24h) or both. Each bar represents the mean value±SEM. *p<0.05, **p<0.01, ***p<0.001 compared to GW4064 treated cells.

The pro-inflammatory cytokines TNFα and IL-1β inhibit FXR transcriptional activity.

In order to investigate whether pro-inflammatory agents (TNFα and IL-1β) decrease FXR target gene expression by inhibiting FXR transcriptional activity, HEK293 cells were transiently transfected with IBABP or SHP reporter constructs alone or in combination with FXR and RXR expression plasmids, and incubated with GW4064 and either TNFα or IL-1β. FXR transcriptional activity of IBABP and SHP promoters was markedly increased by GW4064, while neither TNFα nor IL-1β had any effect on basal luciferase expression. Strikingly, TNFα or IL-1β in combination with GW4064 markedly decreased the IBABP- and SHP-responsive luciferase expression compared to GW4064 alone (Figure 2A and B), suggesting that the inhibition of FXR target gene expression by these pro-inflammatory cytokines is due to a decrease in FXR transcriptional activity.

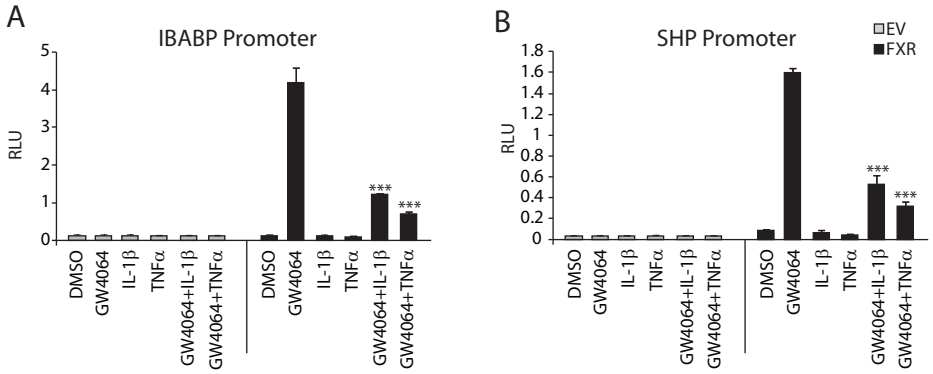

Figure 2: *The pro-inflammatory cytokines TNFα and IL-1β inhibit FXR transcriptional activity.*

Reporter assays of FXR transcriptional activity on IBABP (A) and SHP (B) promoter reporter constructs. Cells were transfected with either empty vector (EV; grey bars) or FXR-RXR (black bars) and treated with DMSO, GW4064 (1μM), TNFα (500U/mL) and IL-1β (20ng/mL) for 24 hours, as indicated. Each bar represents the mean value ± SD; ***p<0.001 compared to GW4064 treated cells.

Overexpression of the NF-κB subunits p50 and p65 inhibits FXR transcriptional activity.
Since TNFα and IL-1β activate NF-κB, we next explored whether NF-κB subunits (p50 and p65) directly affect FXR transcriptional activity. HEK293 cells were transfected with either pGL3-IBABP or pGL3-SHP promoter reporter constructs together with FXR and RXR in combination with either p50, p65 or both. The p50 subunit decreased GW4064-dependent IBABP and SHP promoter activity (by 42% and 85%, respectively) and p65 decreased GW4064-dependent IBABP and SHP promoter activities by 97% and 98%, respectively. Co-transfection of p50 and p65 completely abolished GW4064-dependent IBABP and SHP promoter activity (Figure 3A and B). These results suggest that the inhibition of FXR transcriptional activity is mediated by NF-κB.

FXR interacts with the NFκB subunits p50 and p65 in a GST-pull down assay.
To investigate whether the decrease of FXR transcriptional activity is due to physical interaction between FXR and the NF-κB subunits p50 and p65, a GST-pull down assay was performed. The NF-κB subunits p65 and p50 bind to FXR, in a ligand independent fashion (Figure 3C). Also, GST-pull down assays performed with GST-p65 fusion proteins and [35S]-labeled FXR, revealed interaction between FXR and the NF-κB subunit p65 (Figure 3D).

Fxr activation is inhibited by TNFα and IL-1β in ex vivo ileal samples of WT and Fxr-ko mice.
To extrapolate our *in vitro* findings, we prepared ileal specimens from WT and Fxr-ko mice, and cultured them *ex vivo* in the presence or absence of GW4064 and either TNFα or IL-1β. Fxr expression in the ileal specimens did not change under any of the described conditions (Figure 4A). TNFα and IL-1β treatment alone did not affect Fxr target gene mRNA expression. GW4064 induced the Fxr target genes *Ibabp* and *Fgf15*, only in WT mice. TNFα or IL-1β treatment in combination with GW4064 dramatically reduced *Ibabp* and *Fgf15* expression compared to GW4064 treatment alone (Figure 4B and C), indicating that Fxr activity is reduced.

Fxr activation is decreased in the ileum and colon of mice with DSS-induced colitis.
To explore the inhibition of Fxr activity by inflammatory stimuli *in vivo*, we

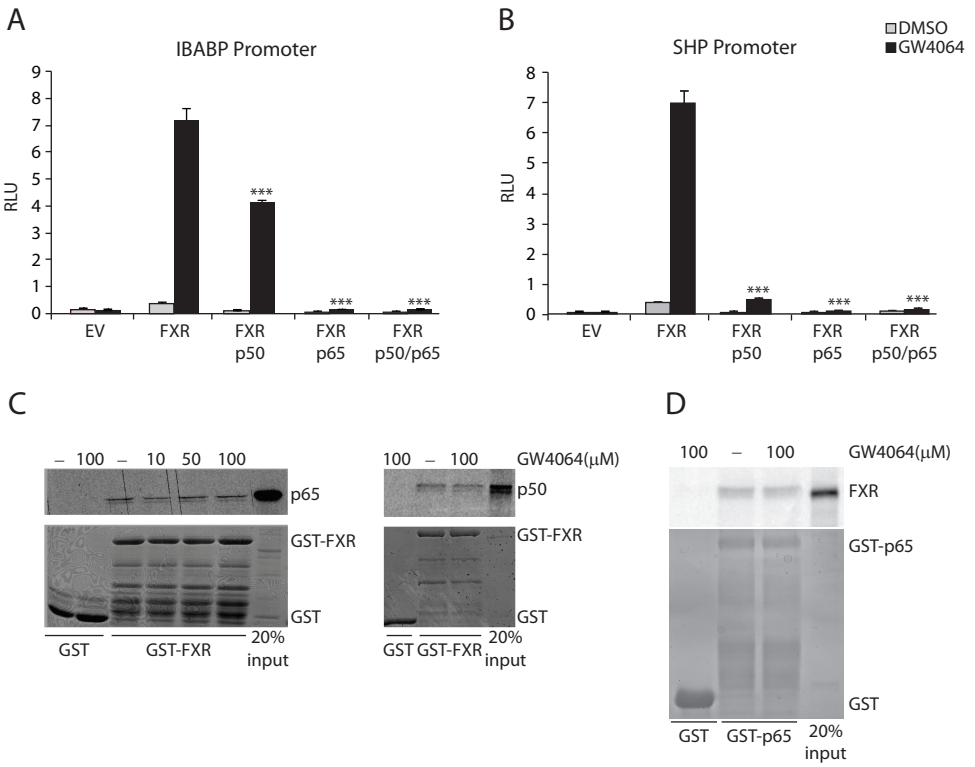

Figure 3: *Overexpression of the NF-κB subunits p50 and p65 inhibit FXR transcriptional activity.*
Reporter assays of FXR transcriptional activity on IBABP (A) and SHP (B) promoter reporter constructs transfected with either empty vector (EV; grey bars) or FXR-RXR (black bars) together with p50, p65 or both. Each bar represents the mean value ± SD; ***p<0.001 compared to GW4064 treated cells. (C) GST-pull down experiment to analyze p50 and p65 binding to FXR. *In vitro* translated p50 and p65 were subjected to a pull-down experiment with GST-FXR and incubated with either DMSO or GW4064, as indicated. The top panel is a radiograph of [35S]-labelled p50 and p65; the lower panel is a Coomassie brilliant blue staining of GST proteins. (D) GST-pull down experiment to analyze FXR binding to p65. *In vitro* translated FXR was subjected to a pull-down experiment with GST-p65 and incubated with either DMSO or GW4064, as indicated. The top panel is a radiograph of [35S]-labelled FXR; the lower panel is a Coomassie brilliant blue staining of GST proteins.

investigated whether DSS-induced inflammation inhibits Fxr-dependent gene expression in the ileal and colonic mucosa of WT and Fxr-ko mice. Fxr was expressed in WT mice, but not Fxr-ko mice. Fxr mRNA expression was not affected by DSS and/or INT747 (Figure 5A and C). Nevertheless, INT-747 activated *Shp*

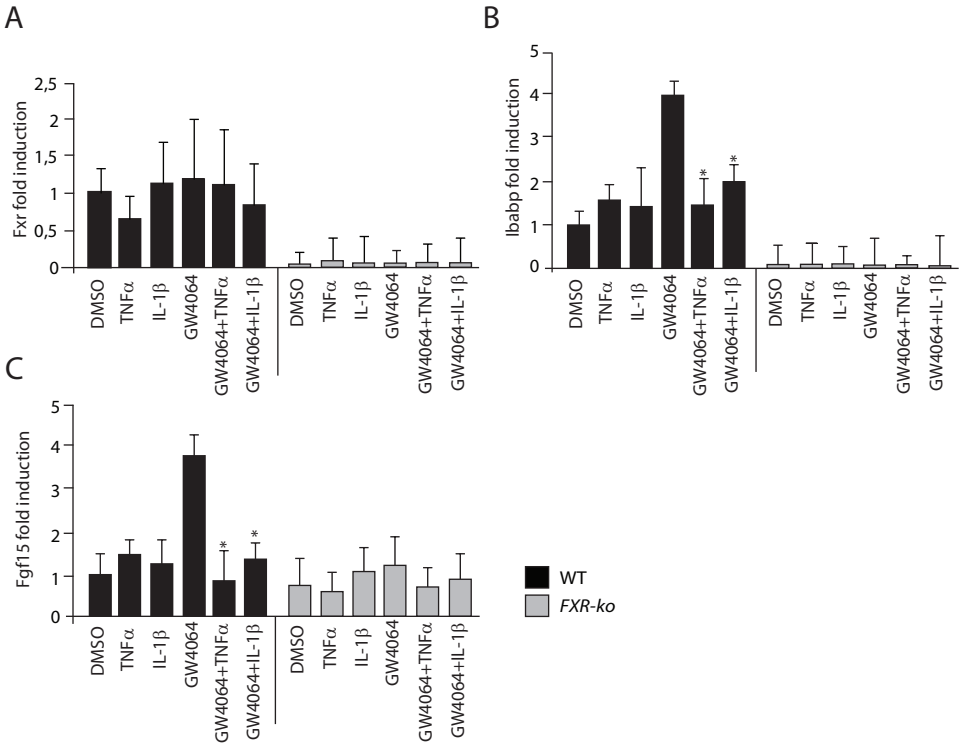

Figure 4: *TNFα and IL-1β decrease Fxr target gene expression in ex vivo cultured mouse ileal mucosa.*

Ileal specimens were prepared from WT and Fxr-ko mice, cultured *ex vivo* and treated for 24 hours with DMSO, GW4064, TNFα and IL-1β, as indicated. mRNA expression of *Fxr* (A) and Fxr target genes *Ibabp* (B) and *Fgf15* (C) in the ileal mucosa of WT (black bars) and Fxr-ko mice (grey bars). Each bar represents the mean value±SEM. *p<0.05 compared to GW4064-treated mice.

mRNA expression in WT, but not in Fxr-ko mice. Concomitant induction of colitis by DSS markedly reduced *Shp* mRNA expression induced by INT-747 in the ileum (Figure 5B). A similar trend was observed in the colon (Figure 5D).

DISCUSSION

We have recently found that FXR is an important player in the counter-regulation of intestinal inflammation. In particular, the potent semi-synthetic FXR agonist INT-747 improves clinical symptoms and histology in the DSS and

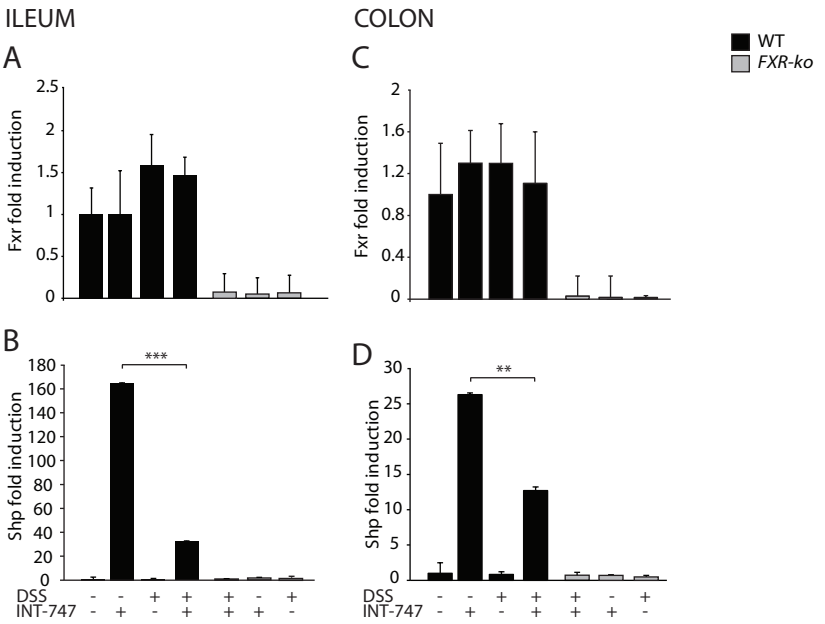

Figure 5: *DSS-induced colitis decreases Fxr activation in WT mice.*
DSS colitis was induced in WT and Fxr-ko mice pre-treated with or without INT-747. mRNA expression of *Fxr* and *Shp* in the ileal (A, B) and colonic mucosa (C, D) of WT (black bars) and Fxr-ko mice (grey bars). Each bar represents the mean value ± SEM. **p<0.01, ***p<0.001 compared to WT INT-747-treated mice.

TNBS murine models of colitis. These beneficial effects are only detected in WT mice, not in Fxr-ko mice. Also, Fxr activation inhibits the increase of epithelial permeability and pro-inflammatory cytokine mRNA expression in the murine intestinal mucosa, under these circumstances[9]. The multi-level protection against intestinal inflammation provides a clear rationale to further explore FXR agonists as a novel therapeutic strategy for IBD. Current treatment options for IBD are mainly aimed at suppressing the immune response. Although reasonably effective, significant side effects and treatment failures can occur, stressing the need for novel treatment options for IBD. Therefore, a therapeutic trial with currently available FXR agonists in patients with inflammatory bowel disease seems warranted, in analogy of currently performed trials with INT-747 in patients with the cholestatic liver disease primary biliary cirrhosis[19]. In the present study, we show mutual crosstalk between FXR and proinflammatory stimuli, because not only does FXR inhibit inflammation, but

also FXR activation is inhibited by pro-inflammatory stimuli in different model systems. Firstly, the pro-inflammatory cytokine TNFα decreases FXR target gene expression in enterocyte-like differentiated HT29 cells. This finding was confirmed in ileal specimens of WT and Fxr-ko mice cultured *ex vivo*, in which we also showed decreased Fxr target gene expression upon TNFα and IL-1β stimulation. Moreover, in mice with severe intestinal inflammation induced by DSS, expression of Fxr target genes *Ibabp* and *Fgf15* was similarly reduced in ileum as well as colon. Although DSS administration leads primarily to colitis, effects on FXR target gene expression in the ileum of DSS-treated mice may be explained by increased levels of circulating pro-inflammatory cytokines originating from the colonic lesions. In line with the data shown in this paper, we have preliminary data indicating that ileal expression of the FXR target gene *SHP* is markedly lower (50%) in patients with Crohn colitis in clinical remission (unpublished data). In Crohn's patients and the *in vitro* and *ex vivo* models presented in this paper FXR expression itself is not significantly changed by pro-inflammatory cytokines, indicating that the inhibition of FXR target gene expression is due to decreased FXR activity. Based on these results, it can be anticipated that ablation of Fxr would lead to more severe intestinal inflammation. Indeed, we observed that Fxr-ko mice have severely impaired intestinal integrity compared to WT mice at baseline (Supplementary Figure 3 of[9]), suggesting that they are probably more susceptible to chronic inflammation. However, we did not find more severe colitis in Fxr-ko mice than in WT mice treated with DSS. This may relate to the short duration of the DSS protocol, the young age of the mice and/or the micro-environmental conditions. It is also possible that the severe inflammation in the DSS model does not allow detecting subtle differences between Fxr-ko and WT mice. This study shows that FXR is not only an active player in inhibition of inflammation, but also is a target of the inflammatory response itself. This could result in a vicious cycle where reduced FXR activity results in less repression of intestinal inflammation, contributing to development of chronic intestinal inflammation. Together, these findings imply an important role for FXR in the protection against IBD. We also hypothesize that decreased FXR activity may lead to altered bile salt enterohepatic circulation and potentially relate to cholestatic liver disease, which often coexists in patients with inflammatory bowel disease[20].

In recent years, a reciprocal regulation between several steroid NRs on the one hand and inflammatory pathways on the other hand has been described. Little is known about the exact mechanisms underlying these processes. For example, NF-κB has been shown to suppress PXR regulated gene expression, by interfering with the binding of PXR to the PXR responsive element[21]. In the current study, we also show direct involvement of NF-κB in repression of FXR activity, since overexpression of NF-κB subunits p50 and p65 inhibits FXR activity and has the ability to physically interact with FXR. Similarly to PXR, NF-κB may inhibit FXR activation by preventing binding of FXR to its cognate FXR responsive element in the DNA. However, at this stage, we cannot rule out other potential mechanisms such as the competition between the nuclear receptor and the NF-κB complex for a common transcriptional cofactor ("cofactor squelching"). It has long been appreciated that inflammation affects bile homeostasis and that bile salts have immune suppressive actions. Although additional mechanisms may exist, our work provides insights in the mechanisms by which bile salts interact with the immune system via FXR.

CONCLUSION

In this paper we show that in addition to the role of FXR in counter-regulation of the intestinal inflammatory response[9], there is reciprocal inhibition of the FXR pathway by the inflammatory response. We provide evidence for involvement of NF-κB in this suppressive effect. The crosstalk between NF-κB and FXR at the intestinal level indicates an interaction between lipid metabolism and inflammatory disease and leads to new insights in pathogenesis and potentially also to novel treatment strategies for inflammatory bowel disease.

Acknowledgment.
We thank Luciano Adorini (Intercept Pharmaceuticals, Corciano, Perugia, Italy) for the FXR ligand INT-747.

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SUPPLEMENTARY TABLE 1: *qRT-PCR Primers (5'-3')*

Human

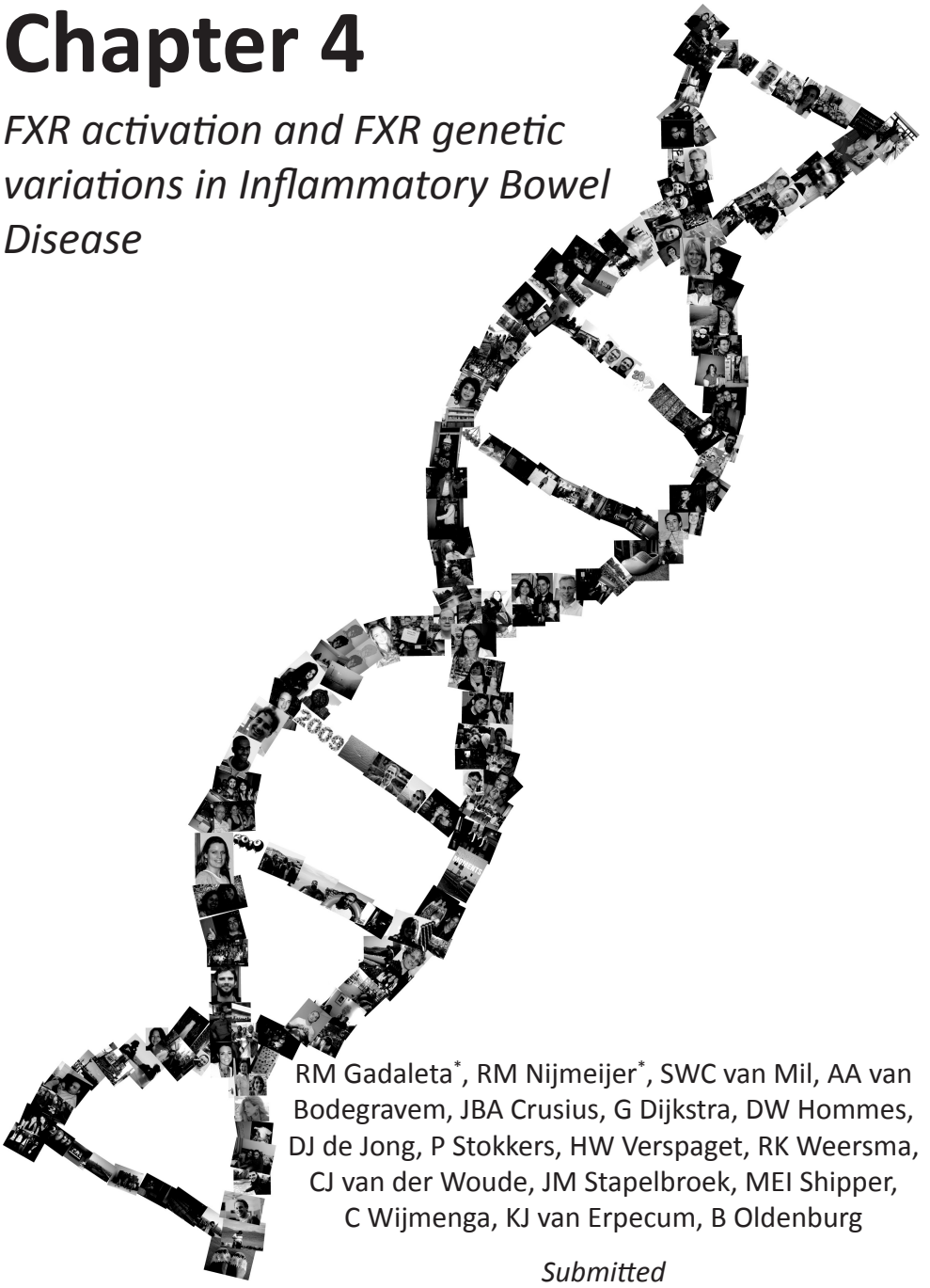
GAPDH F	TGTTGCCATCAATGACCCCTT
GAPDH R	CTCCACGACGTACTCAGCG
FXR F	CTACCAGGATTTCAGACTTTGGAC
FXR R	GAACATAGCTTCAACCGCAGAC
SHP F	AGGGACCATCCTCTTCAACC
SHP R	TTCACACAGCACCCAGTGAG
IBABP F	TCAGAGATCGTGGGTGACAA
IBABP R	TCACGCGCTCATAGGTCA

Mouse

Gapdh F	GCAAAGTGGAGATTGTTGCCAT
Gapdh R	CCTTGACTGTGCCGTTGAATTT
Fxr F	TGAGAACCCACAGCATTTCG
Fxr R	GCGTGGTGATGGTTGAATGTC
Fgf15 F	AAAACGAACGAAATTTGTTGGAA
Fgf15 R	ACGTCCTTGATGGCAATCG
Shp F	CGATCCTCTTCAACCCAGATG
Shp R	AGGGCTCCAAGACTTCACACA

Chapter 4

FXR activation and FXR genetic variations in Inflammatory Bowel Disease



RM Gadaleta*, RM Nijmeijer*, SWC van Mil, AA van Bodegravem, JBA Crusius, G Dijkstra, DW Hommes, DJ de Jong, P Stokkers, HW Verspaget, RK Weersma, CJ van der Woude, JM Stapelbroek, MEI Shipper, C Wijmenga, KJ van Erpecum, B Oldenburg

Submitted

** These authors share first authorship*

ABSTRACT

Background & aims: The bile salt nuclear Farnesoid X receptor (FXR) is expressed in the differentiated enterocyte. We have previously shown that FXR activation protects against intestinal inflammation in mice, but is repressed by the inflammatory stimuli itself. We investigated whether FXR activation is repressed in the ileum and colon of inflammatory bowel disease (IBD) patients. In addition, we hypothesized that genetic variation in FXR may confer susceptibility to IBD and we therefore evaluated whether polymorphisms in the FXR gene are associated with specific phenotypic subgroups of inflammatory bowel disease. ***Methods:*** mRNA expression of *FXR* and FXR target gene *SHP* was determined in ileal and colonic biopsies obtained during screening colonoscopies of healthy controls (n=17), and patients with Crohn's disease (CD; n=15) and ulcerative colitis (UC; n=12) in clinical remission. Seven tagging SNPs and two functional SNPs in FXR were genotyped in a cohort of 2355 Dutch IBD patients (1162 CD and 1193 UC patients) and in 853 healthy controls. ***Results:*** mRNA expression of the FXR target gene *SHP* is significantly reduced in patients with CD but not in patients with UC compared to controls. mRNA expression of the villus marker *Villin* was significantly correlated with *FXR* and *SHP* mRNA expression in healthy controls. However, this correlation was reduced in UC patients and completely lost in CD patients. None of the tagging SNPs was significantly associated with UC or CD. Although the polymorphism 518t>c showed an association with the ileocolonic phenotype of CD, this and other weak associations of different tagging SNPs with colonic or ileocolonic phenotypes disappeared after correction for multiple testing. ***Conclusions:*** FXR activation in the ileum is decreased in patients with CD. This may be secondary to an altered enterohepatic circulation of bile salts or transrepression by inflammatory signals but does not seem to be caused by genetic variation in FXR. The value of increasing FXR activity by synthetic FXR agonists may have potential in the treatment of CD.

INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation and may lead to potentially severe complications and even mortality[1]. Although the exact aetiology is unclear, it is thought to result from a combination of mucosal immune system dysregulation, hyper-reactivity against the intestinal microbiota, and a compromised intestinal epithelial barrier function in genetically predisposed individuals[2]. Genes associated with IBD highlight key pathogenic mechanisms, including disturbed anti-bacterial defence (e.g. NOD2, ATG16L1, cathelicidin, defensins) and barrier function (e.g. PTPsigma, MAGI2, myosin IXB and E-cadherin)[3-6]. Although in recent genome-wide association studies the total number of susceptibility loci amounts to 90, these probably account for only 16% of UC heritability[7] and 20% of CD heritability[8] of the genetic contribution in IBD. It has been estimated that future genome wide association scans will only yield a few more percent of CD heritability. Biological pathway-based analysis or studies focusing on genes involved in established or plausible pathways may be an alternative approach[9]. The bile salt nuclear Farnesoid X Receptor (FXR) is a member of the superfamily of nuclear receptors. Nuclear receptors are ligand-activated transcription factors that, in response to lipophilic ligands (e.g. hormones, vitamins and dietary lipids), regulate many aspects of mammalian physiology, including development, reproduction and metabolism[10,11]. FXR is mainly expressed in the ileum and liver. Upon activation by bile salts, FXR binds as a heterodimer with Retinoid X Receptor (RXR) to the FXR responsive elements on the promoters of target genes, such as the small heterodimer partner (SHP). Via this classical route of transactivation, FXR regulates transcription of genes involved in bile salt synthesis, transport and metabolism in the liver and intestine[12]. FXR has also been implicated in immune modulation and barrier function in the intestine[13,14]. We recently reported that pharmacological FXR activation decreases the severity of inflammation and preserves the intestinal barrier integrity in two well-established murine colitis models[15]. As already described for other nuclear receptors, the mechanism by which FXR affect inflammation is most probably through transrepression of nuclear transcription factor kappa B (NF-κB) signaling. Dysregulated activation of NF-κB was previously identified as a key factor in the pro-inflammatory response in IBD, resulting in strongly enhanced expression of pro-inflammatory genes such as Tumor

Necrosis Factor α or Interleukin 1β and recruitment of excess inflammatory cells to the intestinal wall[16]. Notably, we and others previously showed that FXR is repressed by inflammatory signals via NF-κB *in vitro* and *in vivo*[17,18]. We therefore decided to investigate FXR and FXR target gene mRNA expression in CD and UC in clinical remission. In addition, since FXR acts as a regulator of intestinal inflammation, we hypothesized that also mutations/ functional polymorphisms in FXR might be associated with IBD and tested this hypothesis in a large Dutch cohort of IBD patients and controls.

PATIENTS AND METHODS

Patients.

Seventeen healthy subjects (male/female 7/10; age 55±12.07 years), 15 patients with CD (male/female 5/10; age 46±9.77 years) and 12 patients with UC (male/females 4/8; age 44±9.79 years) were enrolled in this study. All IBD patients were in clinical remission. Patients with significant endoscopic or histological disease activity were excluded. The indication for colonoscopy was screening for cancer or polyps in healthy controls and scheduled dysplasia screening in IBD patients. Biopsies were obtained from the ileum and ascending colon, immediately frozen in liquid nitrogen and subsequently stored at -80°C, until further processing. Informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of the University Medical Centre Utrecht.

mRNA extraction and qRT-PCR analysis.

Human biopsies were homogenized (Omni TH tissue homogenizer, Omni International, Kennesaw, USA) and RNA was isolated using RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The quantity, quality and integrity of isolated mRNA were confirmed by absorption measurement and RNA gel electrophoresis. Subsequently cDNA was generated from 500 ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Roche, Basel, Switzerland). qRT-PCR analysis was carried out using SYBR green PCR master mix (Biorad, Veenendaal, The Netherlands) and a MyIQ real time PCR cycler (Biorad). Values were quantified using

the comparative threshold cycle method. To estimate the distribution between villi and crypts in the human biopsies, we determined mRNA expression of *villin* and *sucrose isomaltase* (*SI*), which are both expressed exclusively in differentiated enterocytes in the villi, and of *c-myc* and *cyclin D1* (*CCND1*), both expressed only in the crypts. mRNA expression levels of genes of interest were normalized to *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*). Primers are listed in Supplementary Table 1.

Patients and controls and the genetic association study.
For the genetic association study, a cohort of 2355 Caucasian IBD patients, consisting of 1162 CD patients and 1193 UC patients was used. This is a subset of a cohort previously described by Weersma and colleagues[19]. Patients were recruited from six university medical centers in the Netherlands (details can be found in Supplementary Table 2). All patients had a confirmed diagnosis of CD or UC, fulfilling standard diagnostic criteria according to clinical, endoscopic, radiological and histological findings[20,21], and were phenotyped according to the Montreal classification[22]. All patients had given written informed consent and all DNA samples and data were handled anonymously. The controls consisted of 853 Dutch blood bank donor controls[23]. All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p<0.05$).

SNP selection and genotyping.
Nine tagging SNPs to cover the complete FXR gene were selected using Haploview[24]. Additionally, two functional SNPs, -1g>t and 518t>c, previously described to affect FXR expression and function[25], respectively, were selected. Two of the tagging SNP assays failed due to technical reasons. With the remaining seven tagging SNPs, 89% of the FXR gene could be tagged with a genetic variance above 3%. Names and chromosomal location of the SNPs are shown in Supplementary Table 3. Genotyping was performed using TaqMan assays on a TaqMan 7900 HT (Applied Biosystems, Foster City, California, USA). All reported p values are uncorrected unless stated otherwise.

Statistical analysis.
Statistical significance was determined by the Student's t-test. Correlation and regression analysis were used to determine the relationships between expression values. Statistical significance for correlation was

determined by Spearman's coefficient test. All statistical calculations were performed with GraphPad Software (SPSS Inc., Chicago, Illinois). Two-sided p-values <0.05 are considered statistically significant. Statistical analysis of the genetic association study was performed using 2-tailed $\chi 2$ tests of case vs. control allele and haplotype counts in Haploview v3.2.[24]; p-values, odds ratios (OR) and 95% confidence intervals (95% CI) are shown. The Bonferroni method was used to correct for multiple testing.

RESULTS

mRNA expression of FXR and its target gene SHP.

FXR and its target gene *SHP* were expressed both in the ileum and ascending colon of IBD patients and controls. Expression levels of *FXR* and *SHP* were markedly lower in the right colon compared to the ileum (53% and 55% lower in the right colon, respectively). There was no significant difference in ileal *FXR* expression between controls, CD and UC patients (Figure 1A). However, ileal expression of *SHP* was 50% lower in CD patients compared to controls (p=0.039), and 33% lower in UC patients compared to controls (p=0.21) (Figure 1B). A similar trend, although not significant, was observed in the colon. To exclude that FXR activation is reduced by a decrease of enterocytes due to ulceration in the biopsies of CD patients, we correlated *FXR* and *SHP* mRNA expression to *villin* expression, a marker exclusively expressed in differentiated enterocytes. *Villin* expression was significantly associated with *sucrose isomaltase* (*SI*, another gene expressed in differentiated enterocytes) expression in controls, UC and CD patients (Figure 6A-C). *Villin* expression was also significantly correlated to *FXR* expression in healthy controls. However, the correlation was lost in UC and CD patients (Figure 6D-F). In addition, *Villin* expression showed significant correlation with *SHP* expression in healthy controls and UC patients, whereas the correlation was lost in CD patients (Figure 6G-I). Similar results were found for the correlation between *SI* expression and either *FXR* or *SHP* (data not shown). The expression of the crypt markers *c-myc* and *CCND1* were significantly correlated. However, *c-myc* and *CCDN1* did not correlate to *Villin*, *SI*, *FXR* or *SHP* expression in any of the groups (data not shown).

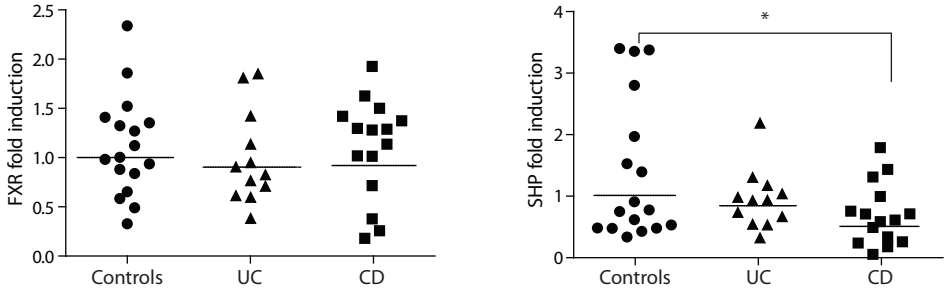


Figure 1: *FXR target gene expression is decreased in patients with Crohn's Disease.*
Scatter plot of mRNA expression of *FXR* and *SHP* in the ileal mucosa of healthy controls (circles), ulcerative colitis (triangles) and Crohn's disease patients (squares). Horizontal lines indicate mean values. Student t-test, *p<0.05 compared to healthy controls.

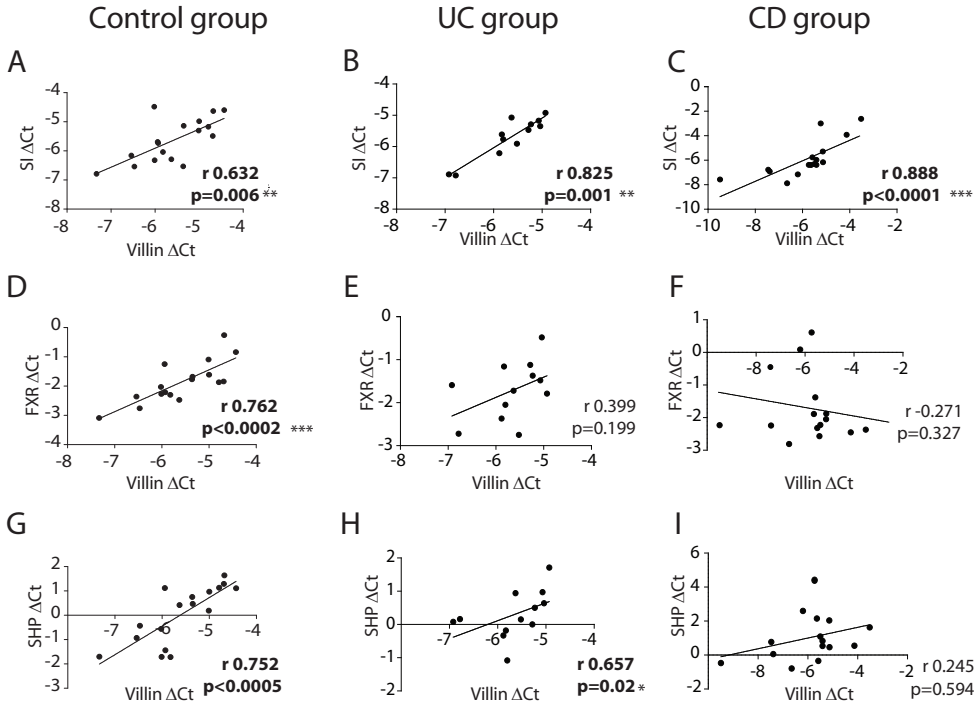


Figure 2: *FXR and SHP correlate with the differentiation marker villin in healthy controls but not in Crohn's Disease patients.*
mRNA expression of *SI*, *FXR* and *SHP* were related by regression analysis to mRNA expression of *Villin* in healthy controls (A, D, G), ulcerative colitis (B, E, H) and Crohn's disease patients (C, F, I). Spearman's coefficient and p values are shown. Values in bold show significant correlations; *p<0.05; **p<0.01, ***p<0.001.

Assessment of FXR genetic variation in IBD patients.
2355 IBD patients and 853 controls were genotyped with seven tagging SNPs and two functional SNPs in FXR. None of the functional SNPs was associated with IBD. However, one of the tagging SNPs displayed a significant association with IBD (rs12313471, p=0.03, OR 1.32, 95% CI 1.02-1.71; Supplementary Table 4). CD (n=1162) and UC patients (n=1193) were also separately compared to the 853 healthy controls. The same tagging SNP (rs12313471) was associated with UC (p=0.048, OR 1.32, 95% CI 1.00-1.76; Supplementary Table 5). None of the SNPs was associated with CD (Supplementary Table 6). None of the above described associations remained significant after Bonferroni correction for multiple testing.

Subgroups analysis.
Phenotypic information on the localization of the disease was present for 1138 of 1162 (98%) patients with CD. We analyzed whether polymorphisms of FXR were associated with CD location using the Montreal classification[22]. Patients with L1 (terminal ileum localization), L2 (colonic localization) and L3 (ileocolonic localization) were compared to the entire CD patients cohort. Two tagging SNPs displayed a significant association with ileal CD (L1; rs11110390, p=0.03, OR 1.26, 95% CI 1.02-1.55 and rs4764980, p=0.03, OR 1.25, 95% CI 1.02-1.53, Supplementary Table 7). None of the SNPs were associated with colonic CD (L2, Supplementary Table 8). Two SNPs showed a significant association with ileocolonic CD (L3), namely the functional SNP 518t>c (p=0.01, OR 3.08, 95% CI 1.08-8.83) and the tagging SNP (rs10860603, p=0.01, OR 1.39, 95% CI 1.07-1.81; Supplementary Table 9). None of these subgroup analysis, however, remained significant after Bonferroni correction for multiple testing.

DISCUSSION

Although the exact aetiology of IBD is not completely understood, several lines of evidence point to an impaired intestinal barrier function and an abnormal immune response in genetically susceptible hosts. Recently, we reported that activation of the nuclear receptor FXR prevents inflammation in animal models of IBD with improvement of colitis symptoms, preservation of the intestinal epithelial barrier function and reduction of goblet cell loss[15].

Furthermore, a negative cross-talk between FXR and the inflammatory response at the intestinal level was demonstrated[17], probably leading to an attenuated intestinal inflammatory status. In the present study, we showed that ileal mRNA expression of the *FXR* target gene *SHP* is markedly reduced in CD patients, whereas FXR expression remained unchanged. This suggests that FXR activity is decreased in this IBD subtype. Previously published genome-wide association scans in IBD patients did not identify loci containing the FXR gene[8]. Since these association studies explain only a small part of the genetic contribution in IBD, we took a candidate approach and studied genetic variation in FXR. In the present study, none of the functional or tagging SNPs proved to be significantly associated with CD or UC. Interestingly, the SNP 518t>c, resulting in the amino acid change M173T, showed an association with the ileocolonic phenotype of CD (Montreal L3)[22]; (p=0.01, OR 3.08, 95% CI 1.08-8.83). This variant has previously been shown to be associated with intrahepatic cholestasis of pregnancy[25], and to result in a 60% decrease in transcriptional activity of FXR. Although the M173T was not significantly associated with CD after correction for multiple testing, it may well be that it plays a modifier role in the aetiology of CD in conjunction with other genes. In addition, other weak associations of different tagging SNPs with colonic or ileocolonic phenotypes disappeared after correction for multiple testing. Thus, a primary genetic defect underlying the role of FXR in CD could not be substantiated. Since the functional SNP 518t>c has a very low prevalence, the possibility of a type II error cannot be excluded. Moreover, two of the selected tagging SNP assays failed due to technical reasons. It therefore cannot be excluded that some SNPs in the remaining 11% of the FXR gene display an association with IBD. Other explanations that could account for the decreased FXR activity in CD should be taken into consideration. This includes the possibility that bile salt uptake in the ileum is reduced, for example due to decreased intestinal transit times. Indeed, several studies have shown increased faecal excretion of bile salts in patients with CD in clinical remission[26-29]. Another mechanism contributing to this phenomenon could be the regulation of bile salt uptake in the ileum. Bile salt uptake and enterohepatic circulation are tightly regulated, and FXR plays a key role in this respect. At the intestinal level, FXR activation increases expression of the ileal bile acid binding protein (IBABP) and basolateral OSTα-OSTβ, allowing negative feedback regulation of bile salt uptake in the ileum. The role of FXR in negative regulation of intestinal

apical bile acid uptake, mediated by the apical sodium-dependent bile acid transporter ASBT, is more complex, with considerable species differences. Nevertheless, FXR probably inhibits ASBT expression in most instances[30-35]. Lastly, reduced FXR target gene expression may be secondary to the reciprocal inhibition of FXR by NF-κB[17,18]. It is well established that a range of pro-inflammatory cytokines is upregulated in the mucosa of IBD patients in remission, potentially resulting in downregulated FXR activity, leading to the observed reduced SHP expression in the current study[36]. In conclusion, we found that FXR expression in the ileum is altered in patients with Crohn's colitis. This could not be explained by the presence of a genetic variation in the FXR gene. Treatment with synthetic FXR agonists may overcome the decrease in FXR activation, possibly resulting in an amelioration of ileocolitis in patients with CD.

Acknowledgments.
We thank Ellen Willemsen for technical assistance and Dr. Leo Klomp for helpful discussion. Prof. Peter Siersema is acknowledge for critically reviewing the manuscript.

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SUPPLEMENTARY TABLE 1: *qRT-PCR Primers (5'-3')*

FXR F	CTACCAGGATTTCAGACTTTGGAC
FXR R	GAACATAGCTTCAACCGCAGAC
SHP F	AGGGACCATCCTCTTCAACC
SHP R	TTCACACAGCACCCAGTGAG
HRPT F	ATTGTAATGACCAGTCAACAGGG
HRPT R	GCATTGTTTTGCCAGTGTCAA
VILLIN F	AGGGCAAGAGGAACGTGGT
VILLIN R	TCCCCTCGGTTGAAACTCTTC
SI F	GGAGATACACCAGAACAAGTAGTTCAA
SI R	AATCCAAGATTCCAATATGCTGG
c-myc F	CCACCACCAGCAGCGACT
c-myc R	CAGAAACAACATCGATTTCTTCCTC
CCDN1 F	CGTGGCCTCTAAGATGAAGGA
CCDN1 R	CGGTGTAGATGCACAGCTTCT

SUPPLEMENTARY TABLE 2: *Patients numbers and hospitals*

Hospital	**Patients**
Academic Medical Centre Amsterdam	439
VU University Medical Centre Amsterdam	647
University Medical Centre Groningen	547
University Medical Centre Leiden	494
St. Radboud University Medical Centre Nijmegen	148
University Medical Centre Utrecht	80
Total number of patients	**2355**

SUPPLEMENTARY TABLE 3: *Name and chromosomal locations of the SNPs tagging FXR*

	SNP number	**Chromosomal location (12)**
Tagging SNPs	rs12313471	100864393
	rs11110390	100874901
	rs4764980	100885107
	rs11110395	100888664
	rs11610264	100932375
	rs10860603	100943948
	rs35739	100948515
Functional SNPs	-1g>t	exon 3
	518t>c	exon 5

Supplementary Table 4: *Association of genetic variants of FXR with the entire IBD cohort (patients with Crohn's disease and ulcerative colitis).*

		IBD patients			Controls			p value*	OR	95% CI
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-g>t	C/A#	4461	125	0.973	1588	36	0.978	0.2674	1.21	0.84-1.76
518t>c	A/G	4545	29	0.994	1616	6	0.996	0.2227	1.52	0.65-3.57
rs12313471	A/G	4245	277	0.939	1548	76	0.953	**0.0317**	1.32	1.02-1.71
rs11110390	C/T	3103	1473	0.678	1070	544	0.663	0.2641	1.07	0.95-1.21
rs4764980	G/A	2261	2271	0.499	832	778	0.517	0.2179	1.07	0.96-1.20
rs11110395	G/T	3182	156	0.953	1538	84	0.948	0.4365	1.12	0.85-1.47
rs11610264	T/C	3141	1343	0.700	1160	458	0.717	0.2138	1.08	0.95-1.23
rs10860603	G/A	3687	549	0.870	1398	214	0.867	0.7492	1.03	0.87-1.22
rs35739	T/C	2441	2033	0.546	900	712	0.558	0.3790	1.05	0.94-1.18

For each Table: OR=odds ratio; 95% CI=95% confidence interval; #Major allele/minor allele; MAF=major allele frequency; *Two-tailed p values were calculated by $\chi 2$ analysis of allele counts; Significant p values are shown in bold.

Supplementary Table 5: *Association of genetic variants of FXR with ulcerative colitis.*

		UC patients			Controls			p value*	OR	95% CI
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-g>t	C/A#	2264	58	0.975	1588	36	0.978	0.5688	1.12	0.74-1.70
518t>c	A/G	2292	10	0.996	1616	6	0.996	0.7549	1.11	0.42-2.95
rs12313471	A/G	2155	141	0.939	1548	76	0.953	**0.0487**	1.32	1.00-1.76
rs11110390	C/T	1605	719	0.691	1070	544	0.663	0.0673	1.14	0.99-1.30
rs4764980	G/A	1116	1182	0.486	832	778	0.517	0.0554	1.13	1.00-1.29
rs11110395	G/T	1340	58	0.959	1538	84	0.948	0.1824	1.26	0.89-1.77
rs11610264	T/C	1585	705	0.692	1160	458	0.717	0.0949	1.13	0.98-1.29
rs10860603	G/A	1769	283	0.862	1398	214	0.867	0.6507	1.04	0.86-1.26
rs35739	T/C	1227	1053	0.538	900	712	0.558	0.2135	1.08	0.95-1.23

Supplementary Table 6: *Association of genetic variants of FXR with Crohn's disease.*

		CD patients			Controls			p value*	OR	95% CI
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-g>t	C/A#	2197	67	0.970	1588	36	0.978	0.1550	1.33	0.88-2.00
518t>c	A/G	2253	19	0.992	1616	6	0.996	0.0725	2.05	0.84-4.99
rs12313471	A/G	2090	136	0.939	1548	76	0.953	0.0548	1.32	0.99-1.76
rs11110390	C/T	1498	754	0.665	1070	544	0.663	0.8845	1.01	0.88-1.16
rs4764980	G/A	1145	1089	0.513	832	778	0.517	0.7954	1.02	0.89-1.16
rs11110395	G/T	1842	98	0.949	1538	84	0.948	0.8636	1.03	0.76-1.39
rs11610264	T/C	1556	638	0.709	1160	458	0.717	0.6024	1.04	0.90-1.20
rs10860603	G/A	1918	266	0.878	1398	214	0.867	0.3153	1.10	0.91-1.34
rs35739	T/C	1214	980	0.553	900	712	0.558	0.7597	1.02	0.90-1.16

Supplementary Table 7: *Association of genetic variants of FXR: subgroup analysis of patients with L1 Crohn's disease vs. Crohn's disease with other location.*

		CD L1 patients			CD patients			p value*	OR	95% CI
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-g>t	C/A#	483	15	0.970	1657	49	0.971	0.8701	1.10	0.61-1.96
518t>c	A/G	503	1	0.998	1690	18	0.989	0.0674	2.83	0.53-14.99
rs12313471	A/G	462	26	0.947	1575	105	0.938	0.4517	1.15	0.74-1.79
rs11110390	C/T	309	187	0.623	1147	551	0.676	**0.0294**	1.26	1.02-1.55
rs4764980	G/A	279	221	0.558	840	834	0.502	**0.0273**	1.25	1.02-1.53
rs11110395	G/T	400	26	0.939	1393	71	0.952	0.3021	1.30	0.82-2.06
rs11610264	T/C	348	138	0.716	1166	486	0.706	0.6625	1.05	0.84-1.31
rs10860603	G/A	436	48	0.901	1431	211	0.871	0.0830	1.32	0.95-1.84
rs35739	T/C	273	211	0.564	896	754	0.543	0.4139	1.09	0.89-1.33

Supplementary Table 8: *Association of genetic variants of FXR: subgroup analysis of patients with L2 Crohn's disease vs. Crohn's disease with other location.*

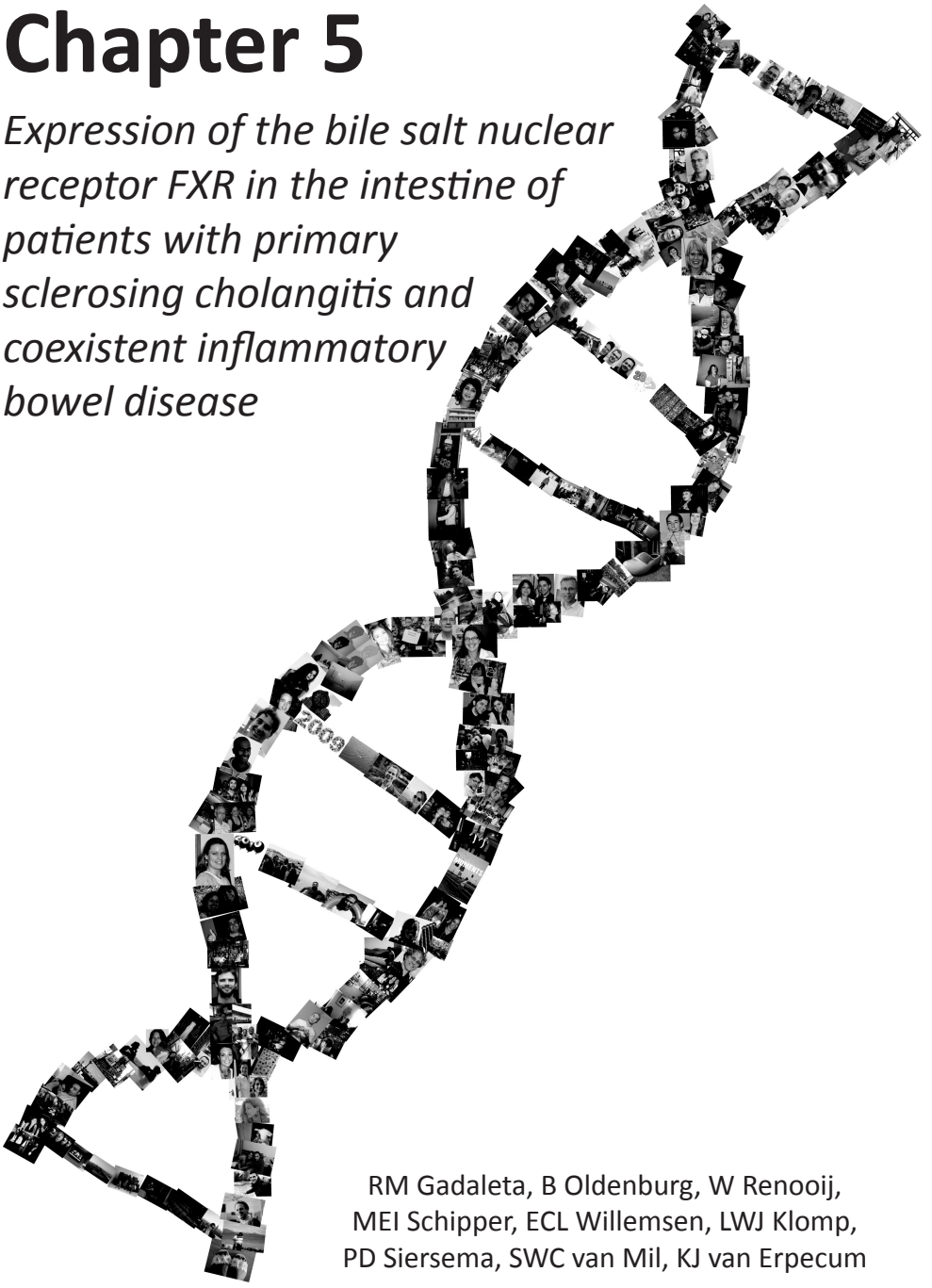
		CD L2 patients			CD patients			p value*	OR	95% CI
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-g>t	C/A#	561	17	0.971	1579	47	0.971	0.9503	1.05	0.60-1.84
518t>c	A/G	573	3	0.995	1620	16	0.990	0.3065	1.50	0.47-4.79
rs12313471	A/G	538	28	0.951	1499	103	0.936	0.2032	1.29	0.84-1.97
rs11110390	C/T	388	184	0.678	1068	554	0.658	0.3870	1.09	0.89-1.34
rs4764980	G/A	293	275	0.516	826	780	0.514	0.9502	1.01	0.83-1.22
rs11110395	G/T	472	30	0.940	1321	67	0.952	0.3174	1.27	0.82-1.98
rs11610264	T/C	395	173	0.695	1119	451	0.713	0.4366	1.09	0.88-1.34
rs10860603	G/A	489	59	0.892	1378	200	0.873	0.2394	1.19	0.88-1.62
rs35739	T/C	304	258	0.541	865	707	0.550	0.7029	1.04	0.86-1.26

Supplementary Table 9: *Association of genetic variants of FXR: subgroup analysis of patients with L3 Crohn's disease vs. Crohn's disease with other location.*

		CD L3 patients			CD patients			p value*	OR	95% CI
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-g>t	C/A#	1096	32	0.972	1044	32	0.970	0.8481	1.05	0.64-1.72
518t>c	A/G	1117	15	0.987	1076	4	0.996	**0.0150**	3.08	1.08-8.83
rs12313471	A/G	1037	77	0.931	1000	54	0.949	0.0806	1.37	0.96-1.95
rs11110390	C/T	759	367	0.674	697	371	0.653	0.2879	1.10	0.92-1.31
rs4764980	G/A	547	559	0.495	572	496	0.536	0.0558	1.18	1.00-1.39
rs11110395	G/T	921	41	0.957	872	56	0.940	0.0808	1.43	0.95-2.16
rs11610264	T/C	771	313	0.711	743	311	0.705	0.7479	1.03	0.86-1.24
rs10860603	G/A	942	152	0.861	925	107	0.896	**0.0130**	1.39	1.07-1.81
rs35739	T/C	592	496	0.544	577	469	0.552	0.7276	1.03	0.87-1.22

Chapter 5

Expression of the bile salt nuclear receptor FXR in the intestine of patients with primary sclerosing cholangitis and coexistent inflammatory bowel disease



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Submitted

ABSTRACT

Background: Farnesoid X receptor (FXR) is a bile salt nuclear receptor expressed in the differentiated enterocyte and hepatocyte, involved in pathogenesis of Crohn and cholestatic liver disease. ***Methods:*** mRNA expression of *FXR* and its target gene *SHP* were determined in ileal biopsies of 15 patients with primary sclerosing cholangitis (PSC) and coexistent inflammatory bowel disease (IBD): 9 Crohn-like colitis, 6 ulcerative colitis, (all on UDCA therapy) and 17 controls. Bile salt and phospholipid molecular species composition were determined by HPLC in duodenal bile of 8 PSC patients, before and after 3 weeks ursodeoxycholic acid treatment (UDCA, 750 mg/day). ***Results:*** There were no significant differences in ileal *FXR* and *SHP* expression between controls and PSC-IBD patients. mRNA expression of the villus marker Villin significantly correlated to mRNA expression of *FXR* and its target gene *SHP* in healthy controls but not in PSC-IBD patients. At baseline, the primary bile salts cholate and chenodeoxycholate were most abundant, with minor contribution of secondary bile salt deoxycholate and resulting hydrophilic bile salt composition. During treatment, UDCA comprised approximately 30% of total bile salts, with further increased hydrophilicity. 16:0–18:2 phosphatidylcholine was the most abundant phospholipid, followed by 16:0-18.1 and 16:0-20:4 phosphatidylcholine, without appreciable changes during UDCA therapy. ***Conclusion:*** Expression of FXR and its target gene SHP is not decreased in patients with PSC and coexistent IBD, despite hydrophilic bile salt pool enriched in UDCA. Potential therapeutic effects of currently available powerful FXR agonists in this patient category remain to be explored.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation. It is thought to result from a combination of dysregulation of the mucosal immune system, hyperreactive against the intestinal microbiota, and compromised intestinal epithelial barrier function in genetically predisposed individuals[1]. There is ample evidence that the ileum is a key location where prevention of excessive intestinal inflammation and maintenance of intestinal barrier are orchestrated. For example, endogenous antimicrobial peptides such as α-defensins are produced in the ileum, and their levels are reduced in Crohn's disease, thereby compromising mucosal host defence[2]. Also, phospholipid concentration and composition in colonic mucus layer (pivotal in intestinal barrier function) are dependent on bile salt-induced phospholipid secretion in the ileum with subsequent spread to the distal colon by propulsory motility, and these are deficient in patients with IBD[3,4]. Finally, recent data indicate that the bile salt nuclear Farnesoid X Receptor (FXR) is crucial for prevention of intestinal inflammation[5]. FXR is mainly expressed in the ileum and liver. Once activated by bile salts, it binds as a heterodimer with Retinoid X Receptor (RXR) to FXR-responsive elements on the promoters of target genes, such as the small heterodimer partner (SHP), intestinal bile acid binding protein (IBABP) and fibroblast growth factor 15/19 (FGF15/19 in mouse and human, respectively), involved in bile salt homeostasis[6]. Bile salts are involved in maintaining intestinal barrier integrity: in the rodent bile duct ligation (BDL) model and in humans with bile duct obstruction, bile salt deficiency in the intestine is associated with mucosal injury, bacterial overgrowth and translocation. Oral administration of bile salts counteracts these deleterious effects[7-9]. Insufficient activation of FXR may be the link between intestinal bile salts and intestinal barrier function: Fxr-ko mice exhibit compromised intestinal integrity, with further deterioration after BDL, which is not prevented by Fxr agonist treatment. In contrast, FXR agonists prevent mucosal injury, bacterial overgrowth and translocation after bile duct ligation in wild type mice[10]. We have recently found that pharmacological FXR activation decreases the severity of inflammation and preserves the intestinal barrier integrity in two well-established murine colitis models[5]. Also, we have shown that ileal expression of the FXR target gene *SHP* is markedly lower (50%) in patients with Crohn's colitis in clinical remission

(without coexistent liver disease) than in controls or patients with ulcerative colitis (see Chapter 4). Dysregulated activation of the nuclear transcription factor κappa B (NF-κB) was previously identified as a key factor in the pro-inflammatory response in IBD, resulting in strongly enhanced expression of pro-inflammatory genes such as *Tumor Necrosis Factor α* or *Interleukin 1β* and recruitment of excess inflammatory cells to the intestinal wall[11]. Reciprocal inhibition of FXR and NF-κB has been demonstrated, and may be the underlying mechanism for the beneficial effects of FXR activation in IBD[5,12,13]. A significant proportion of IBD patients are suffering from primary sclerosing cholangitis (PSC). The most frequent type of IBD in PSC patients is thought to be ulcerative colitis[14-16]. Nevertheless, inflammatory bowel disease in PSC patients is characterized by a unique phenotype with extensive colitis, rectal sparing, ileal inflammation and mild clinical symptoms[14-17]. This "Crohn-like" phenotype in PSC patients could be associated with decreased FXR expression, similar to our previous findings in Crohn's disease patients without PSC (see Chapter 4). In addition, decreased amounts of intestinal bile salts due to the cholestatic liver disease could lead to less FXR activation in the enterocyte. Also, treatment with the bile salt ursodeoxycholic acid (UDCA, generally prescribed in this patient category) could lead to more hydrophilic bile salt composition and again, less intestinal FXR activation, since UDCA is a poor agonist for FXR compared to the major endogenous bile salts chenodeoxycholate (CDCA), cholate (CA) and deoxycholate (DCA)[18]. One may hypothesize that altered FXR expression/activation in ileal cells could also provide the missing link between the intestinal and liver disease in PSC patients, since FXR activation is also considered pivotal for prevention of cholestatic liver disease[19-21]. In fact, beneficial effects of treatment with the powerful FXR agonist 6-ethylchenodeoxycholic acid (INT747: Obeticholic acid®, Intercept Pharmaceuticals Inc, New York, NY) have been demonstrated on liver biochemistry in patients with the cholestatic liver disease primary biliary cirrhosis, and phase 3 trials are currently being performed in this patient category[22]. In the current work we explore gene expression levels of *FXR* and its target gene *SHP* in ileal biopsies of patients with PSC and coexistent IBD and explore biliary bile salt composition before and during UDCA treatment. Since phospholipid acyl chain composition profoundly affects behavior of bile salts in mixed micellar solutions[23], we also determine biliary phosphatidylcholine molecular species composition.

PATIENTS and METHODS

FXR expression in ileum.

Ileal biopsies were obtained during colonoscopy from 15 PSC patients (nine with Crohn-like disease and six with ulcerative colitis) and 17 healthy subjects. Results from the control groups have been reported before (See Chapter 4). Diagnosis of Crohn-like colitis was based on discontinuous pattern of inflammation, relative rectal sparing, minor histologic activity in ileal biopsies and occurrence of granulomas[14]. All patients exhibited clinically quiescent disease and at most mild inflammation during endoscopy and subsequent histological analysis. All used 5-aminosalicylic acid (1000-3000 mg/day) and ursodeoxycholic acid (750-1500 mg/day). One patient was also on immune suppressive therapy with prednison and azathioprine (7.5 mg and 100 mg/day, respectively) and another patient was using adalimumab (Humira®: 40 mg/2 weeks), both of them in combination with the above described medications. The indication for colonoscopy was a cancer screening programme for the healthy controls and regular dysplasia screening for the IBD-PSC patients. Biopsies were obtained from the ileum, immediately frozen in liquid nitrogen and subsequently stored at -80°C, until further processing. Informed consent was obtained from all subjects and the study was approved by the Ethical Committee of the University Medical Centre of Utrecht.

mRNA extraction and qRT-PCR analysis.

Human biopsies were homogenized (Omni TH tissue homogenizer, Omni International, Kennesaw, USA) and RNA was isolated using RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The quantity, quality and integrity of isolated mRNA were confirmed by absorption measurement and RNA gel electrophoresis. Subsequently, cDNA was generated from 500 ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Roche, Basel, Switzerland). qRT-PCR analysis was carried out using SYBR green PCR master mix (Biorad, Veenendaal, The Netherlands) and a MyIQ real time PCR cycler (Biorad). Values were quantified using the comparative threshold cycle method. mRNA expression of *FXR* and its target gene *SHP* were studied. FXR and its target genes are exclusively expressed in the differentiated enterocyte in the villi[10]. To estimate the distribution between villi and crypts in the human biopsies, we determined mRNA expression of *Villin* and *sucrose isomaltase*

(*SI*), which are both expressed exclusively in the differentiated enterocytes in the villi, and *c-myc* and *cyclin D1* (*CCND1*), both expressed only in the crypts. mRNA expression levels of genes of interest were normalized to *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*). Primers are listed in Table 1.

TABLE 1: *qRT-PCR Primers (5'-3')*

FXR F	CTACCAGGATTTCAGACTTTGGAC
FXR R	GAACATAGCTTCAACCGCAGAC
SHP F	AGGGACCATCCTCTTCAACC
SHP R	TTCACACAGCACCCAGTGAG
HRPT F	ATTGTAATGACCAGTCAACAGGG
HRPT R	GCATTGTTTTGCCAGTGTCAA
VILLIN F	AGGGCAAGAGGAACGTGGT
VILLIN R	TCCCCTCGGTTGAAACTCTTC
SI F	GGAGATACACCAGAACAAGTAGTTCAA
SI R	AATCCAAGATTCCAATATGCTGG
c-myc F	CCACCACCAGCAGCGACT
c-myc R	CAGAAACAACATCGATTTCTTCCTC
CCDN1 F	CGTGGCCTCTAAGATGAAGGA
CCDN1 R	CGGTGTAGATGCACAGCTTCT

Biliary bile salt composition before and after ursodeoxycholic acid treatment. In a separate group of eight fasting PSC patients, bile was collected by duodenal intubation after slow intravenous injection of the cholecystokinine analog ceruleine (Takus®, Farmitalia, Milan, Italy) both before and at the end of three weeks treatment with ursodeoxycholic acid (Ursofalk®, Tramedico, Weesp, The Netherlands) 750mg/day ingested a.n.. Patients had not received ursodeoxycholic acid at least one month before the first measurement. Diagnosis of PSC was based on liver biopsy and endoscopic retrograde pancreatico-cholangiography (ERCP) in all patients. Patient characteristics were as follows: male/female ratio 6/2, age (mean±SD) 38±12 years, intrahepatic and combined intrahepatic-extrahepatic disease in 50% each, early and late histological stage (stage 1-2 vs 3-4 according to Ludwig)[24] in 50% each, associated IBD in 75% of cases. Conjugated bile salt species were analyzed in the whole bile by isocratic high performance liquid chromatography (Shimadzu HPLC, SPD-6AV) using a Supelcosil 5µm column LC-18-DB (25cm x 4.6 mm). Methanol/phosphate buffer (23.5

mM sodium phosphate in 70 % methanol) was used as solvent (pH 5.2; flow rate, 1 mL min−1) with detection at 200 nm[25]. Phospholipid classes were isolated by thin-layer chromatography on silica gel (60-HR; Merck, Darmstadt, Germany) with chloroform/methanol/acetic acid/water (50/25/8/2.5, v/v/v/v) as a solvent system[26], and extracted from the silica via a Bligh and Dyer system[27]. Phosphatidylcholine was separated into its molecular species according to acyl chain length and degree of unsaturation, as described by Kito *et al.*[28]. Phosphatidylcholines were converted into their respective diglycerides, derivatized with dinitrobenzoyl chloride and analyzed at 254 nm by high-performance liquid chromatography (Shimadzu HPLC, SPD-6AV) on Supelcosil 5µm column LC-18-DB using acetonitril-isopropanol (80/20, v/v) as solvent at a flow rate of 1 mL/min. Cumulative hydrophobicity index for bile salt species was calculated according to Heuman[29], and for phosphatidylcholine species according to Angelico et *al.*[30].

Statistical analysis.

Results are expressed as mean ±SEM or ±SD, as indicated in the figure legends. All statistical calculations were performed with GraphPad Software (SPSS Inc., Chicago, Illinois). Statistical significance was determined by ANOVA, paired or unpaired T-tests, as appropriate. Correlation and regression analyses were used to determine the relationships between expression values. Statistical significance for correlation and linear regression was determined by r Spearman's coefficient. Two-sided p-values <0.05 are considered statistically significant.

RESULTS

FXR and FXR target gene expression in ileum.

Patient characteristics of the PSC-IBD groups undergoing ileal biopsy are given in Table 2. Patients with Crohn-like colitis tended to exhibit more advanced liver disease than patients with ulcerative colitis. There was no significant difference in ileal *FXR* and *SHP* mRNA expression between the controls and PSC patients with Crohn-like colitis or ulcerative colitis (Figure 1A and B). FXR and its target genes are exclusively expressed in the differentiated enterocyte in the villi. Nevertheless, results were similar when corrected for various amount of villi versus crypts in the ileal biopsies (data not shown).

Table 2: *Patient characteristics in the groups with primary sclerosing cholangitis and coexistent Crohn-like disease or ulcerative colitis.*

	PSC+Crohn-like Colitis	PSC+UC
Nr. patients	9	6
Intra-/Extrahepatic/Combined	1/0/8 (12%/0%/88%)	2/0/4 (33%/0%/67%)
Histological state (1-2 vs 3-4)	3/6 (33% vs 67%)	4/2 (67% vs 33%)
Bilirubin	30±24 (26; 10-86)	16±6 (14; 9-40)
AP	305±108 (270; 161-476)	298±228 (237; 106-689)
γGT	282±233 (162; 58-708)	149±82 (139; 55-270)
ASAT	63±23	58±48
ALAT	76±26	74±58
Albumin	36±6	37±4
Trombocyten	212±94 (161; 114-374)	261±51 (278; 190-321)
PT	13.1±0.8	13.5±1.4

Results are given as mean±SD. In case of non-parametric distribution, medians and range are also shown in parentheses.

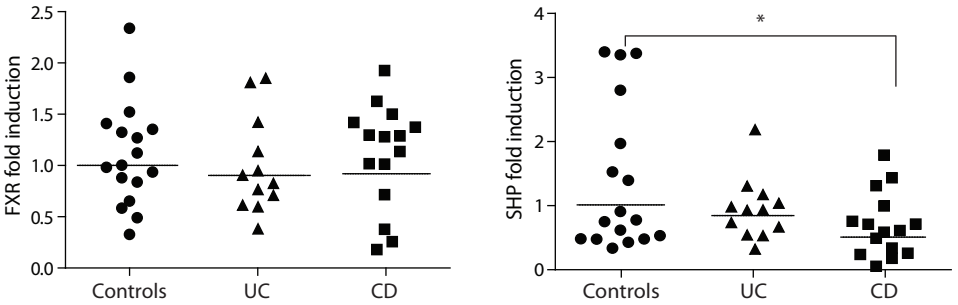

Figure 1: *FXR and SHP gene expression is not different in primary sclerosing cholangitis patients with Crohn-like disease, ulcerative colitis or controls.*
Scatter plot of mRNA expression of *FXR* (A) and *SHP* (B) in the ileal mucosa patients with primary sclerosing cholangitis and Crohn-like disease (squares), with primary sclerosing cholangitis and ulcerative colitis (triangles) or healthy controls (circles). Horizontal lines indicate mean values.

Also, there was no correlation between serum bilirubin levels and mRNA expression of *FXR* or *SHP* in the total group or in the three subgroups (data not shown). In order to further examine the expression level of *FXR* and *SHP* in ileal biopsies of IBD patients, a correlative analysis was conducted. *Villin* and *Sucrose Isomaltase* (*SI*) expression levels were chosen to represent differentiated enterocytes in ileal biopsies. *Villin* expression was significantly associated with *SI* expression in controls and PSC patients with Crohn-like colitis (Figure 2A and B). *Villin* expression was also significantly correlated to *FXR* as well as *SHP* expression in healthy controls (Figure 2 D and G). However

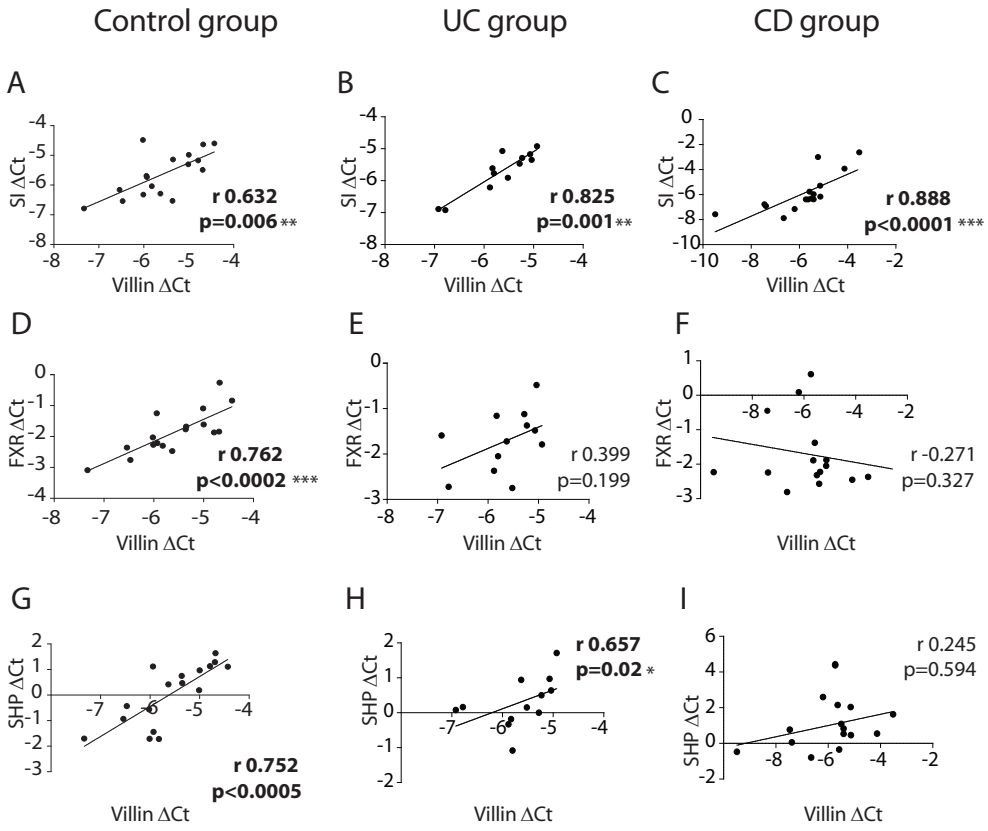

Figure 2: *FXR and SHP expression levels correlate with the differentiation marker villin in healthy controls but not in the group of primary sclerosing cholangitis and coexistent Crohn-like disease.*

mRNA expression of *SI*, *FXR* and *SHP* were related by regression analyses to mRNA expression of *Villin* in healthy controls (A, D, G), primary sclerosing cholangitis with coexistent Crohn-like disease (B, E, H) and primary sclerosing cholangitis with coexistent ulcerative colitis (C, F, I). Values in bold indicate significant correlations.

the correlation lost significance in PSC patients with Crohn-like colitis (Figure 2E and H). The analysis for *Villin* versus *SI*, *Villin* versus *FXR* and *Villin* vs *SHP* expression levels in PSC patient with ulcerative colitis did not show any significant correlation, probably due to the small number of patients in this group. Similar results were found for the relationship between *SI* expression and either *FXR* or *SHP* (data not shown). The expression of the crypt markers *c-myc* and *CCND1* were significantly correlated. However, *c-myc* did not correlate to *Villin*, *SI*, *FXR* or *SHP* expression in any of the groups (data not

shown). Similar results (i.e. absence of correlation) were found for the relationship between *CCDN1* and either *FXR*, *SHP*, *villin* or *SI* (data not shown).

Biliary bile salt and phosphatidylcholine molecular species composition.
At baseline, the primary bile salts cholate and chenodeoxycholate were the most abundant bile salts, with a minor contribution of the secondary bile salt deoxycholate to the bile salt pool (Table 3). As a result, cumulative bile salt

Table 3: *Bile salt species (%) in patients with primary sclerosing cholangitis, at baseline and after 3 weeks ursodeoxycholic acid therapy.*

Bile Salt Species (%)	Before URSO	After URSO	p value	*Reference values(32)
Glycocholate	36.5±3.0	28.2±2.8	p=0.012	26.7±1.2
Taurocholate	24.1±4.9	10.3±1.8	p=0.017	14.0±1.4
Glycochenodeoxycholate	19.6±4.0	17.8±1.2	p=0.63	24.4±1.2
Taurochenodeoxycholate	10.5±1.8	5.6±0.6	p=0.005	10.8±0.9
Glycodeoxycholate	5.2±1.4	4.1±1.5	p=0.47	17.5±2.1
Taurodeoxycholate	3.4±2.2	1.0±0.2	p=0.32	5.0±0.5
Glycolithocholate	0.1±0.1	0.1±0.1	p=0.59	0.21±0.1
Taurolithocholate	0.3±0.1	0.2±0.1	p=0.41	0.22±0.1
Glycoursodeoxycholate	1.8±0.4	28.0±2.3	p=0.000002	0.78±0.2
Tauroursodeoxycholate	0.5±0.2	3.7±0.4	p=0.00001	0.20±0.1
Glyco/Tauro Ratio	2.4±1.5	4.1±1.2	p<0.05	3.2±0.4
Cumulative hydrophobicity Index	0.20±0.07	0.03±0.04	p<0.001	0.34±0.02

Results are given as mean±SEM. *Reference values from patients with multiple cholesterol gallstones.

hydrophobicity index was quite hydrophilic [0.2±0.07 (mean±SEM), compared to approximately 0.36 in gallstone patients or normal controls][31,32]. At the end of the treatment period, ursodeoxycholic acid comprised approximately 30% of total bile salts, with further increased hydrophilicity as indicated by a cumulative bile salt hydrophobicity index of 0.03±0.04 (mean±SEM). At baseline, most bile salts were glycine-conjugated, which was even more pronounced during ursodeoxycholic acid (ratio glycine/taurine conjugated bile salts 2.4±1.5 and 4.1±1.2, respectively). Regarding phosphatidylcholine molecular species composition, 16:0–18:2 phosphatidylcholine was most abundant, followed by 16:0-18:1 and 16:0-20:4 phosphatidylcholine, in line with previous reports in gallstone patients and healthy subjects (Table 4)[31,33].

Table 4: *Phosphatidylcholine molecular species (%) in patients with primary sclerosing cholangitis, at baseline and after 3 weeks ursodeoxycholic acid therapy.*

Phosphatidylcholine molecular species	Before URSO	After URSO	p value	Reference value gallstone pts (33)
16:0-22:6	5.8±1.1	6.7±0.6	p=0.25	5.3±0.2
16:0-20:4	8.1±1.3	7.7±1.0	p=0.22	11.3±0.6
18:1-18:2	2.5±0.3	3.4±0.2	p=0.0016	3.4±0.2
16:0-18:2	47.9±2.8	50.9±1.3	p=0.69	57.1±0.8
18:0-20:4	1.6±0.4	1.7±0.3	p=0.11	1.7±0.1
18:1-18:1	0.3±0.1	0.7±0.1	p=0.09	0.4±0.1
16:0-18:1	32.3±4.8	27.5±0.9	p=0.27	20.0±0.7
16:0-16:0	0.5±0.2	0.4±0.1	p=0.28	0.5±0.1
18:0-18:2	0.1±0.1	0.1±0.1	p=0.053	not detected
18:0-18:1	1.0±0.3	0.9±0.2	p=0.51	0.3±0.1
Cumulative hydrophobicity Index	44.3±2.2	41.3±0.5	p=0.27	45.2±0.2

Results are given as mean±SEM.

Table 5: *Liver biochemistry values in patients with primary sclerosing cholangitis, at baseline and after 3 weeks ursodeoxycholic acid therapy.*

	Before URSO treatment	After URSO treatment	p value
Bilirubin	15±6 (14; 7-24)	11±4 (10; 7-16)	p=0.11
AP	355±194 (290; 103-628)	240±107 (241; 88-382)	p=0.24
γGT	553±480 (290; 214-1551)	293±246 (189; 71-650)	p=0.07
ASAT	62±37	63±50	p=0.90
ALAT	101±66	89±73	p=0.53

Results are given as mean±SD. In case of non-parametric distribution, medians and range are also shown in parentheses.

The proportion of 16:0-18.2 phosphatidylcholine was somewhat lower and the proportion of 16:0-18:1 phosphatidylcholine somewhat higher in the PSC-IBD patients when compared with a previously reported disease control group (patients with cholesterol gallstones) determined with the same methodology[33], but similar to previously reported normal subjects (determined with a slightly different methodology)[31]. Most importantly, considering their profound effects on mixed micelle formation, phosphatidylcholine species with disaturated acyl chains remained to form a virtually negligible proportion of phosphatidylcholine molecular species. There were no major changes in phosphatidylcholine molecular species composition during

ursodeoxycholic acid therapy, although liver biochemistry tended to improve during ursodeoxycholic acid, albeit without reaching significance (Table 5).

DISCUSSION

The bile salt nuclear receptor FXR in the ileum has recently been implicated as a key player in intestinal barrier function and prevention of bacterial overgrowth and translocation[10]. We have recently found that pharmacological FXR activation decreases the severity of inflammation and preserves the intestinal barrier integrity in two well-established murine colitis models[5]. We also found a significant decrease in mRNA expression (50% reduction) of the FXR target gene *SHP* in ileal biopsies of patients with clinically and histologically quiescent Crohn colitis (without coexistent liver disease). In contrast *SHP* mRNA expression in ileal biopsies of patients with ulcerative colitis (without coexistent liver disease) proved to be the same as in healthy controls (see Chapter4). Up to 90% of patients with the cholestatic liver disease primary sclerosing cholangitis (PSC) exhibit inflammatory bowel disease, generally characterized by a unique phenotype with extensive colitis, rectal sparing, ileal inflammation and mild clinical symptoms[14-17]. One may hypothesize that altered FXR expression in the enterocyte could also provide the missing link between the intestinal and liver disease in PSC patients, since FXR activation is considered pivotal for prevention of cholestatic liver disease[19-21]. In fact, phase 3 trials are currently performed with the powerful synthetic FXR agonist 6-ethylchenodeoxycholic acid (INT747: Obeticholic acid®, Intercept Pharmaceuticals Inc, New York, NY) in patients with the cholestatic liver disease primary biliary cirrhosis[22]. The major finding of the current work is that there appears to be no change in expression of *FXR* or its target gene *SHP* in patients with PSC and coexistent Crohn-like colitis. There are several points that need to be considered. First, ileal biopsies were obtained during colonoscopy, after bowel preparation and in the fasting state. We therefore cannot exclude that results would have been different during feeding with enhanced bile salt enterohepatic circulation. Nevertheless, our previous study revealed significantly lower expression of the FXR target gene SHP in patients with Crohn's disease without coexistent liver disease, with ileal biopsies obtained after bowel preparation and under the same fasting

circumstances. Second, since FXR and its target genes are only expressed in the differentiated enterocytes in the villi[10] we correlated expression of *villin* (villus marker) and *c-myc* (crypt marker) to expression of *FXR* and *SHP*. Interestingly, there was a significant correlation between *villin* and *FXR* as well as *SHP* expression in normal controls, but this correlation was lost in the PSC patients with Crohn-like colitis. Although these data would suggest altered FXR expression/activity in PSC patients with coexistent Crohn-like disease, the implications of these findings are not clear and further research is needed. Third, biliary bile salt composition is expected to be different in patients with cholestatic liver disease, and this might be even more pronounced during treatment with the hydrophilic bile salt ursodeoxycholic acid which was prescribed to all PSC patients in the current study. We therefore performed in a separate group of PSC patients, a detailed evaluation of the effects of cholestatic liver disease and the influence of ursodeoxycholic acid on biliary bile salt composition. The primary bile salts cholate and chenodeoxycholate were most abundant under basal circumstances, with virtual disappearance of the secondary bile salt deoxycholate, which is normally formed from cholate by bacterial 7-α dehydroxylase activity in the intestine. As a result, bile salt hydrophobicity was more hydrophilic than in disease controls[32] or normal subjects[31] from previous reports. Treatment with ursodeoxycholic acid induced a further increase in bile salt hydrophilicity. These data are in line with our previous reports on bile salt composition in PSC patients, performed with different methodology (Gas-Liquid Chromatography rather than HPLC)[34]. As far as FXR expression is concerned, one would expect if anything, decreased expression of FXR and target genes under these circumstances, since ursodeoxycholate is a poor ligand of FXR[18]. Fourth, biliary phosphatidylcholine acyl chain composition has profound effects on bile salt incorporation in mixed micelles[23]. The principal phospholipid in the gastrointestinal tract is bile-derived phosphatidylcholine, which exceeds diet-derived phosphatidylcholine by as much as 5:1[35]. Phosphatidylcholine in human bile normally contains mainly 16:0 acyl chains at the sn-1 position and mainly unsaturated (18:2>18:1>20:4) acyl chains at the sn-2 position[31]. Most of this phosphatidylcholine is digested by pancreatic phospholipase-A2[36-38]. In presence of bile salts, phosphatidylcholine with saturated acyl chains (e.g. dipalmitoyl phosphatidylcholine) exhibits much lower affinity for mixed micelle formation with bile salts and cholesterol (with preferential

formation of cholesterol-phospholipid vesicles) than phosphatidylcholine with unsaturated acyl chains[23]. Also, phospholipase-A2 induced hydrolysis is much lower under these circumstances[39]. As a result, phosphatidylcholine acyl chain composition could profoundly affect the bile salt intermicellar-vesicular (non-phospholipid associated) fraction available for absorption in the enterocyte[40,41]. We previously found altered phospholipid species composition in bile of patients with benign recurrent intrahepatic cholestasis[42], and a similar phenomenon could in theory occur in patients with PSC. Although relative amounts of biliary phosphatidylcholine versus bile salts have been reported to be normal in PSC[43], there are no data on phosphatidylcholine acyl chain composition available in this patient group. In the current work, we found only minor changes, of uncertain significance, in phosphatidylcholine molecular species, compared to disease controls or normal subjects[31,33]. Most importantly, considering their profound effects on mixed micelle formation, phosphatidylcholine species with disaturated acyl chains remained to give a virtually negligible contribution to phosphatidylcholine molecular species. In conclusion, we found in the current work no evidence that expression of *FXR* or its target gene *SHP* is decreased in patients with primary sclerosing cholangitis and coexistent Crohn-like colitis, despite hydrophilic bile salt pool enriched in ursodeoxycholic acid. Potential therapeutic effects of the currently available powerful FXR agonists in this patient category remain to be explored.

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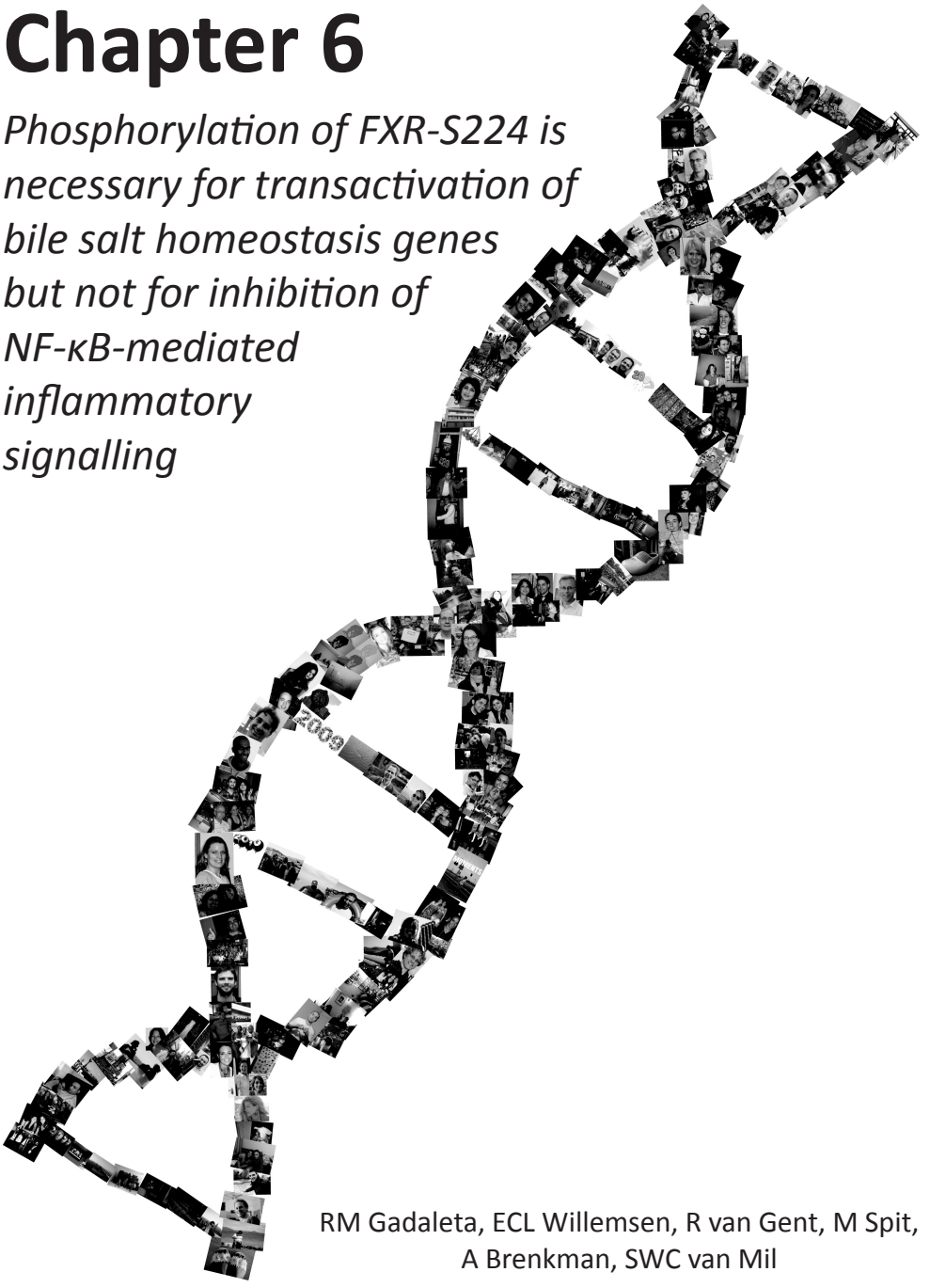
Chapter 6

Phosphorylation of FXR-S224 is necessary for transactivation of bile salt homeostasis genes but not for inhibition of NF-κB-mediated inflammatory signalling



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Manuscript in preparation

ABSTRACT

The bile salt nuclear Farnesoid X receptor (FXR) is a key regulator of bile salt, cholesterol, lipid and glucose metabolism via classical transactivation of gene expression. In addition, we have recently shown that FXR also inhibits the inflammatory response, likely via NF-κB transrepression, as similarly to other nuclear receptors such as PXR, PPARs and GR. It is generally accepted that different modifications and different cofactors are necessary for NRs to transactivate and transrepress gene transcription. Here we aimed to start dissecting the molecular pathways regulating FXR transactivation and transrepression. HEK293T cells transfected with FLAG-FXRα2 were treated with or without GW4064 for 24 hours. Subsequently, we performed FLAG-immunoprecipitations and ascertained sites of phosphorylation by Orbitrap mass spectrometry. Phosphorylation was detected at Serine 224 (S224). Substitution of S224 into S224A (defective in phosphorylation) resulted in abrogated transcriptional activity of FXR on SHP, IBABP and BSEP promoters in reporter assays. In contrast, transactivation capacity of FXR-S224D (phospho-mimicking mutant) was similar or higher than wild type (WT) FXR. Electro-mobility shift assays show absence of binding of FXR-S224A to BSEP, SHP, and IBABP oligos, in contrast to FXR-WT and FXR-S224D. Heterodimerization of FXR-S224A to RXR is unaffected as shown by GST-pull down assays. Interestingly, the ability of FXR-S224A to inhibit NF-κB signalling by transrepression is unaffected in reporter assays. ***Conclusion:*** Phosphorylation of FXR-S224 is important for gene target transactivation but not NF-κB transrepression *in vitro*. These findings potentially advance drug design for FXR, since selective FXR ligands which do not result in S224 phosphorylation may be useful to treat hepatic/intestinal inflammation without interfering with bile salt, glucose and fat metabolism.

INTRODUCTION

Nuclear receptors (NRs) are ligand-activated transcription factors that regulate transcription of a great variety of target genes. NRs function by transactivation of target genes via binding to consensus NR-response elements (NREs). As such, NRs mediate many physiological processes, including reproduction, metabolism, and development[1,2]. In addition, it has been recognized that several members of the NR family have physiological roles as negative regulators of inflammatory responses. For this, NRs function independent of direct DNA-binding, by transrepression, in which NRs interfere with the gene regulation of other promoter-bound transcription factors(e.g. AP-1, NF-κB), resulting in amelioration of inflammatory status[3]. NRs are unique transcription factors because their activity is regulated by specific ligands that easily pass biological membranes, making them ideal drug targets. Although a striking 13% of all FDA-approved drugs target a NR, serious side effects limit their utility and safety[4]. This is generally accepted to be due to general activation of all transcriptional actions of the NR (transactivation and transrepression). For example, synthetic glucocorticoid receptor agonists, such as prednisolone, have potent anti-inflammatory activities, but also induce many adverse effects when used chronically, including hypertension, glucose intolerance and muscle weakness. It is widely accepted that these side effects are mostly attributed to the ability of GR to induce transcription of target genes by transactivation[5]. There is, therefore, a great interest in developing selective modulators of NR action that exert anti-inflammatory effects but exhibit a lower incidence of undesirable side effects than full agonists. Identification of the molecular mechanisms responsible for the anti-inflammatory actions of NR ligands is thus likely to aid in the efforts to develop safer and more effective drugs. NRs undergo several post-translational modifications (phosphorylation, acetylation, sumoylation, etc.) and interact with many components of the transcriptional machinery. However, how these modifications and interacting proteins contribute to transcriptional changes that lead to different biological responses is largely unknown. Farnesoid X receptor (FXR, NR1H4) is activated by endogenous bile salts, hormone-like molecules that circulate between the liver and intestine and are crucial for the uptake of dietary fats and vitamins from the intestinal lumen. Bile salts are highly toxic, and it was discovered that FXR is the master regulator of bile salt concentrations in cells by regulating the inward and

outward bile salt transport systems, bile salt metabolism and the *de novo* synthesis from cholesterol in the liver. FXR modulates the expression of its target genes by heterodimerizing with the retinoid X receptor (RXR, NR2B1) to FXR response elements (FXREs) in the DNA. FXR not only regulates bile salt homeostasis, but also cholesterol, fat and glucose metabolism (reviewed in [6]). We have recently found that also FXR is an important player in the counter-regulation of intestinal inflammation[7]. In particular, the potent semi-synthetic FXR agonist INT-747[8] improves clinical symptoms and histology in the DSS and TNBS murine models of colitis. These beneficial effects are only detected in wild type (WT) mice, not in Fxr-ko mice. Also, Fxr activation inhibits the increase of epithelial permeability and pro-inflammatory cytokine mRNA expression in the murine intestinal mucosa under these circumstances[7]. The multi-level protection against intestinal inflammation provides a clear rationale to further explore FXR agonists as a novel therapeutic strategy for inflammatory bowel diseases. However, side effects regarding the homeostasis of bile salts, glucose and cholesterol are anticipated when full FXR agonists will be used to treat intestinal inflammation. Here, we aimed to start dissecting the molecular pathways regulating FXR transactivation and transrepression essential to improve rational FXR drug design and evaluation.

METHODS

Phospho-site identification.

Cells were transfected with pEBB-Flag- FXRα2 and pcDNA-RXR plasmids. At day 2, cells were incubated with DMSO or 1μM of the FXR synthetic agonist GW4064 (Sigma-Aldrich, Saint Louis, MO, USA). After 24 hours, cells were lysed in buffer containing 20mM Tris pH 8,1% Triton, 0.5% Sodium Deoxycholate (NaDOC), 0.1% SDS, 10mM EDTA, 150mM NaCl, complete protease inhibitor cocktail tablet (Roche, Woerden, The Netherlands), supplemented with 5mM sodium fluoride (NaF) to preserve phosphorylations. Lysates were first pre-cleared for 1 hour with protein A-agarose beads (Roche) and subsequently incubated with Anti-Flag M2 agarose beads (Sigma-Aldrich) for 2 hours at 4°C. Afterwards, immuno-precipitated protein fractions in 1x Laemli Sample Buffer were separated by Nupage® Novex® 4-12% Bis-

Tris minigel according to manufacturer's instructions, and stained with SimplyBlue Safe-stain (Invitrogen, Carlsbad, CA, USA). Bands were excised from gel, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Roche) as described previously[9]. Samples were subjected to nanoflow LC (Eksigent, Dublin, Ireland) using an online C18 Reverse phase-TiO_2-Reverse phase column to enrich phosphorylated peptides as described previously[10] followed by a C18 Reverse phase analytical column (Reprosil; column dimensions, 20 cm x 50 µm; packed in-house). Column eluate was directly coupled to a LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operating in positive mode, using Lock spray internal calibration. Data were processed and subjected to database searches using MASCOT software (Matrixscience, London, UK) against Swiss Prot and non-redundant NCBI database, with a 10ppm mass tolerance of precursor and 0.5Da for the fragment ion.

Plasmids.

In order to generate FXR protein mutated at the Serine in position 224, site-directed mutagenesis of the pcDNA3.1-hFXRα2 was carried out according to manufacturer's protocol (Quick Change site directed mutagenesis Kit, Stratagene, La Jolla, CA, USA) using the primers in Table 1. We generated an FXR mutant with disruption of the phospho-site, substituting the Serine at position 224 with an Alanine (FXR-S224A). Additionally, a phospho-mimicking FXR mutant, substituting the same Serine with an Aspartate, was created (FXR-S224D). Constructs sequences were verified by sequence analysis. Primers are listed in Table 1. In addition, by substituting a Leucine at position 434 with an Arginine, we generated an FXR mutant defective in RXR binding (FXR-L434R), analogous to the homodimerization-defective mouse ER mutant (ER-L511R).[11] All other plasmids have been described before[7,12].

Table 1: *Primers used for site-directed mutagenesis of pcDNA3.1-FXRα2*

hFXRα2-L434R FW	cactttgcctgtctccggggtcgcctgactgaattacgg
hFXRα2-L434R RW	gtgaaacggacagaggccccagcggactgacttaatgcc
hFXRα2-S224D FW	gtgaatgaagacgctgaaggtcgtgac
hFXRα2-S224D RW	gtcacgaccttcagcgtcttcattcac
hFXRα2-S224A FW	gtgaatgaagacgatgaaggtcgtgac
hFXRα2-S224A RW	gtcacgaccttcatcgtcttcattcac

Cell culture.
Human embryonic kidney cells HEK293T were grown at 37°C with 5% CO2 in Dulbecco Modified Eagle's Medium (DMEM) GlutaMax™ (4.5 g/L D-Glucose and Pyruvate; Gibco BRL, Breda, the Netherlands), supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Gibco BRL). HEK293 cells were treated with DMSO, PBS, the synthetic FXR ligand GW4064 (1uM for 24 hours), the natural FXR ligand Chenodeoxycholic acid (CDCA, 10μM, for 24 hours), Tumor Necrosis Factor α (TNFα; 500U/mL, for 24h), as indicated in the figure legends.

Reporter assays.
HEK293T cells were grown in 96-multiwell plates and co-transfected with empty pGL3-IBABP, pGL3-SHP, pGL3-BSEP or pGL3-2κB reporters, tK-Renilla or CMV-Renilla and either empty pcDNA or pcDNA-hFXRα2 wild type (FXR-WT) or mutant, as indicated in the figure legend, together with pcDNA-RXRα, using the standard calcium phosphate method, as described elsewhere[13]. After 24 hours, cells were incubated with DMSO, GW4064 and TNFα, as indicated. Cells were lysed after 24 hours and Firefly and Renilla luciferase activity were measured according to manufacturer's instructions (Promega Dual-Luciferase Reporter Assay System, Promega, Madison, Wisconsin, USA) with the Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium).

Immuno blotting.
HEK293T cells were transfected with hFXRα2 expression plasmids and harvested after 48 hours. Protein lysates or *in vitro* translated proteins (used in EMSA) were electro-transferred to nitrocellulose membranes and were probed with anti-FXR antibody (1:1000, PPMX, Tokyo, Japan). Immunoreactivity was detected with horseradish peroxidase-conjugated antibodies and chemiluminescence (DAKO, Glostrup, Denmark).

Electro-mobility shift assays.
FXR-WT, FXR-S224A, FXR-S224D and RXRα proteins were *in vitro* translated, using the TNT Quick Coupled Transcription/translation system (Promega, Madison, WI). Binding reactions contained 25 mM Hepes pH 7.9, 1mM EDTA, 0,5mM EGTA, 5% glycerol, 1% NP-40 50 mM NaCl, 20mMDTT, 1 μg of poly(dI-dC) and 2 μl of each *in vitro* translated protein. Samples were pre-incubated at

room temperature for 5 minutes prior to the addition of ^{32}P-labeled double-stranded oligonucleotide probe (0.2 pmol; probe sequences are listed in Table 2). Where indicated, α-FXR antibody or cold specific or non-specific (PPARγ RE) probes were added to the pre-incubation mix at a 12-fold molar excess. Samples were held at room temperature for further 30 minutes, and the protein-DNA complexes were resolved on a pre-electrophoresed 5% polyacrylamide gel in 0.5 X TBE. ^{32}P-labelled probes were detected by autoradiography and analyzed with Storm 820 apparatus (Molecular dynamics, Pharmacia Biotecnology, Amersham Biosciences, Diegem, Belgium).

Table 2: *Sequences of FXRE probes used in EMSAs.*

hBSEP	5'-GCCCTTAGGGACATTGATCCTTAGGCAA-3'
hIBABP	5'-GATCGGCCAGGGTGAATAACCTCGGGG-3'
mSHP	5'-GCCTGGGTTAATGACCCTGTT-3'

GST-pull down.

Generally, GST-pull down experiments were performed as described elsewhere.[12] Briefly, FXR expression plasmids were transcribed and translated *in vitro* in the presence of ^{35}S-methionine and incubated with GST, or GST-RXR-LBD fusion proteins coupled to gluthatione sepharose beads. Samples were subsequently washed, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Coomassie brilliant blue was used to visualize GST proteins. ^{35}S-labelled proteins were detected by autoradiography and analyzed with Storm 820 apparatus (Molecular dynamics, Pharmacia Biotechnology, Amersham Biosciences, Diegem, Belgium).

RESULTS

FXR-S224A, but not FXR-S224D, displays abrogated transactivation capability.

In order to identify novel post-translational modifications in FXRα2, we subjected immunoprecipitated Flag-FXR fractions to highly sensitive Orbitrap mass spectrometry. Next to a previously identified Threonine phosphorylation site (T442)[14], we identified phosphorylation of the Serine in the position 224 (S224, table 3). S224 and the surrounding amino acids are conserved in mammals and are positioned in the hinge domain of FXR (Figure 1). We generated an FXR mutant with disruption of the phospho-site , substituting

Homo Sapiens	KSKRLRKNVKQHADQTVNED-SEGRDLRQVTSTTKSCREKTE
Mus Musculus	KSKRLRKNVKQHADQTANEDDSEGRDLRQVTSTTKFCREKTE
Bos Taurus	KSKRLRKNVKQHADQAIHED-SEGRDLRQVTSTTKSCREKTE
Rattus Norvegicus	KSKRLRKNVKQHADQTVNED-SEGRDLRQVTSTTKLCREKTE
Equus Caballus	KSKRLRKNVKQHADQTINED-SEARDLRQVTSTTKSCREKTE
Consensus	KSKRLRKNVKQHADQt.nED.SEgRDLRQVTSTTKsCREKTE

Figure 1: *Serine 224 in FXR is located in the Hinge region.*
Species alignment of the amino acid sequence around the S224 residue in the Hinge domain. The position of S224 is indicated (shaded).

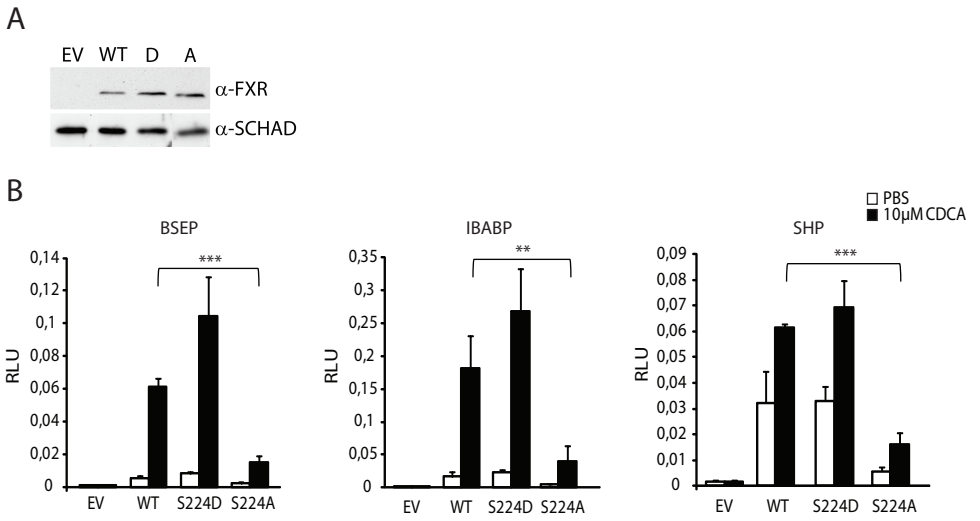

Figure 2: *FXR-S224A, but not FXR-S224D, displays abrogated transactivation capability.*
(A) Immunoblot analysis of HEK293 cells transfected with FXR-WT, FXR-S224D and FXR-S224A plasmids; (B) HEK293T cells were cotransfected with pcDNA3.1 (EV), FXR WT or FXR S224A/D expression plasmids together with RXR and the pGL3-BSEP, pGL3-SHP or pGL3-IBABP promoter reporter constructs and treated with PBS (white bars) or CDCA (10μM, black bars) for 24 hours, as indicated. Subsequently, luciferase expression was assayed. Each bar represents the mean value±SD; **p<0.01, ***p<0.001 compared to PBS treated cells.

Table 3: *Identified phospho-peptides through LC-Orbitrap MS/MS analysis.*
Immunoprecipitated FXR protein was digested and subjected to TiO_2 purification to enrich for phosphorylated peptides. In total, 44% of FXR was identified using various digestive enzymes and a single phosphosite was identified.
** Protein * * Entry name as in UniprotKB/Swiss-prot database; † Accession number according to UniprotKB/Swiss-prot database; ‡ Number of unique peptides identified by MS/MS; § Sequence coverage based on MS/MS analysis*

Protein*	Acc nr†	Size (kDa)	AA	Sequence	Missed cleavage	Mascot score
FXR (NR1H4)	**Q96RI1**	55	225-241	NVKQHADQTVNEDSEGR	1	54
FXR (NR1H4)	**Q96RI1**	55	225-244	NVKQHADQTVNEDSEGRDLR	2	37
FXR (NR1H4)	**Q96RI1**	55	228-244	QHADQTVNEDSEGRDLR	1	31

the Serine at position 224 with an Alanine (FXR-S224A). Additionally, a phospho-mimicking FXR mutant, substituting the same Serine with an Aspartate, was created (FXR-S224D). Although the S224A/D mutants display similar expression levels as FXR-WT (Figure 2A), in reporter assays, the S224A mutant results in a significant reduction of BSEP, SHP and IBABP promoter activity upon treatment with the endogenous FXR ligand CDCA (Figure 2B). In contrast, the phospho-mimicking mutant with the S224D mutant showed similar or increased transactivation ability compared to FXR-WT (Figure 2B). These results suggest that phosphorylation of FXR at S224 is necessary for *in vitro* FXR-dependent transactivation of its target gene promoters.

FXR-S224 and FXR-S224D display unaltered transrepression of NF-κB signalling.

In order to investigate whether phosphorylation of S224 is also important for the transrepression of NF-κB transcriptional activity (previously shown in [7]), HEK293 cells were transfected with a κB reporter plasmid and RXR, in combination with either empty vector (EV), FXR-WT, FXR-S224A or FXR-S224D, and incubated with TNFα, CDCA or both. In cells transfected with the κB-reporter alone, TNFα increased NF-κB activity. CDCA had no effect on basal luciferase expression and on TNFα-mediated NF-κB activity. In cells co-transfected with FXR-WT or S224D mutant, CDCA decreases the TNFα-induced κB-responsive

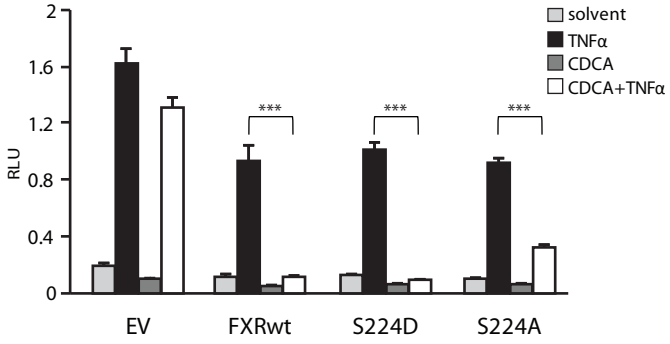

Figure 3: *FXR-S224A and FXR-S224D display unaltered transrepression of NF-κB signalling.* HEK293T cells were co-transfected with pcDNA3.1 (EV), FXR-WT or FXR-S224A/D expression plasmids together with RXR and the pGL3-2xκB promoter reporter construct and treated with PBS (white bars) or CDCA (10μM, black bars) and/or TNFα for 24 hours, as indicated. Subsequently, luciferase expression was assayed. Each bar represents the mean value±SD; ***p<0.001 compared to PBS treated cells.

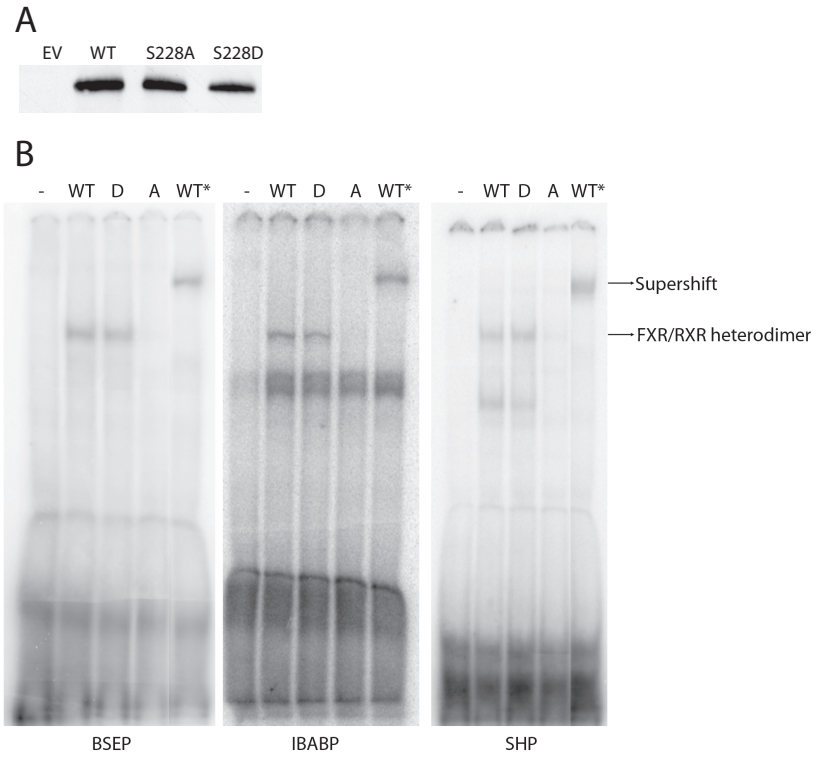

Figure 4: *FXR-S224A but not FXR-S224D is defective in DNA binding to FXREs.*
(A) Western Blot of *in vitro* translated EV, FXR-WT, FXRS224A/D protein probed with anti-FXR antibody. (B) EMSA using *in vitro* translated FXR WT, FXR-S224D, FXR-S224A and RXR proteins and a radiolabeled oligonucleotide corresponding to the IR-1 FXR responsive element from BSEP, IBABP and SHP promoters. The FXR/RXR heterodimeric complex, as well as the super-shifted complex are indicated. (WT= FXR-WT; A=FXR-S224A; D=FXR-S224D; *in these lanes, 1µl of FXR-antibody was added).

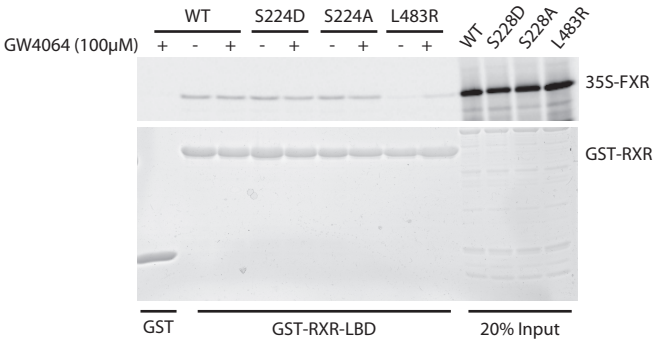

Figure 5: *FXR-S224A and FXR-S224D display unaltered RXR heterodimerisation in vitro.*
GST pull-down experiments were performed to analyze FXR binding to RXR. *In vitro* translated FXR proteins (WT and mutant) were incubated with or without 100μM GW4064 and subjected to a pull-down experiment with bacterially expressed and purified GST-RXR. The top panel is a radiograph of ^{35}S-labeled FXR proteins; the lower panel shows Coomassie brilliant blue staining of GST proteins.

luciferase expression. The S224A mutant also significantly repressed NF-kB activity, albeit less pronounced. This data suggest that phosphorylation of S224 is not essential for transrepression of NF- κB signalling (Figure 3).

FXR-S224A but not FXR-S224D is defective in DNA binding to FXREs.
In order to further characterize the mechanism by which S224 regulates transactivation, we next investigated DNA binding properties of FXR-WT, FXR-S224A and FXR-S224D mutants by electromobility shift assays. *In vitro* translated RXR and FXR proteins (either WT or mutant,) were equally expressed and incubated with ^{32}P-labeled FXRE sequences from the SHP, BSEP and IBABP promoters (Figure 4A). A specific FXR-RXR heterodimeric complex was formed on the FXRE because formation of this protein-DNA complex could be diminished by addition of an excess of unlabeled WT FXRE, and was not present when a mutant SHP probe was used (Supplementary Figure 1). In addition, specific antibodies against FXR (Figure 4B) and RXR (data not shown) supershifted the WT protein-DNA complexes, confirming the heterodimeric composition of the complexes. These results suggest that phosphorylation of S224 is necessary for DNA binding and transactivation of FXR target genes.

FXR-S224A and FXR-S224D display unaltered RXR heterodimerization in vitro.
To investigate whether the decrease of FXR-S224A transcriptional activity is due to the loss of binding capacity to RXR, a GST-pull down assay was performed. For this, *in vitro* translated ^{35}S-methionine-labeled FXR proteins (WT or mutants) were incubated with purified GST-RXR. As a control, a FXR mutant was generated (L434R), analogous to the homodimerization-defective mouse ER mutant (L511R)[11]. FXR-L434R mutant showed an abrogated ability of binding to RXR in GST-pull down assays (Figure 5). FXR-WT, FXR-S224D and FXR-S224A showed physical interaction with RXR, (Figure 5). The same results were obtained when ^{35}S-methionine-labeled RXR proteins were incubated with bacterially expressed and purified GST-FXR (WT or mutant, data not shown). These results suggest that the *in vitro*

heterodimerization of FXR to RXR is not depending on phosphorylation of S224.

DISCUSSION

Classical FXR action involves transactivation of genes involved in bile salt homeostasis, cholesterol, fat and glucose metabolism[6]. However, recently we also showed that FXR regulates transcription in a different manner, by transrepression. FXR activation counteracts pro-inflammatory cytokine expression and secretion by enterocytes and different human immune cell types via transrepression of NF-κB signalling[7]. It is eminent that the activity of FXR is dynamically tailored towards distinct physiological stimuli in these two different modes of transcriptional action. These differential molecular mechanisms are however largely unknown. The general mechanism by which Nuclear Receptors initiate transcription is by releasing co-repressor (CoR) and recruiting co-activator (CoA) complexes upon ligand binding. This CoA complex modifies the chromatin structure, yielding access to general transcription factors and RNA polymerase II to promoter DNA. About 300 different cofactors for NRs are described, some of which have been implicated in FXR-mediated transcription (DRIP205, CARM-1, PRMT-1, PGC1α, etc.15-18). However, these studies show the requirement for FXR-mediated transcription of these cofactors on only a single promoter *in vitro*, and need further research to define their *in vivo* necessity. Moreover, most cofactor-FXR interactions were established in a biased manner by testing whether cofactors already identified as partners for other NRs were binding partners for FXR. It is very likely that other (yet unidentified) proteins might modulate FXR transcriptional activity *in vivo* more significantly. In addition, the role of FXR in regulating inflammation is caused not by transactivating, but by transrepressive actions of FXR, and this will eminently be caused by different cofactor interactions and post-translational modifications. Recently, it has been established that FXR can be phosphorylated, acetylated and sumoylated[19-21]. However, it is not known whether these modifications are specific for transactivation or whether they also occur during transrepression. Here, we aimed to take an unbiased approach to identify FXR post-translational modifications for the different contexts (transactivation and transrepression) in which FXR

operates. We show for the first time, that a post-translational modification is regulating transrepression and transactivation of a NR differently. By means of mass spectrometry, we identified a novel phosphorylation site in FXR, S224. The S224A (phospho-defective) mutant displays abrogated transactivation of SHP, IBABP and BSEP promoters, whereas the S224D (phospho-mimicking) mutant has unaltered transactivation capacity. Both mutants are capable to transrepress NF-κB activity. The S224 residue is positioned in the hinge region of FXR, a region that is known to be important for DNA binding of NRs in general[22]. Indeed, we show that FXR-S224A is defective in DNA binding in EMSAs. These results suggest that phosphorylation of the S224 residue is important for binding to FXREs and concurrently transactivation capability. Future studies are aimed to further dissect the molecular mechanisms of transactivation and transrepression. The first step to pursue this study is to identify the kinase responsible for S224 phosphorylation. In addition, phospho-specific antibodies are being generated to be able to study FXR-S224 phosphorylation *in vivo*. These findings potentially advance drug design for FXR, since selective FXR ligands which do not result in S224 phosphorylation may be useful to treat hepatic and intestinal inflammation without interfering with bile salt, glucose and fat metabolism.

Acknowledgement.
We thank Aukje Veentstra for her expert mass spectrometry assistance.

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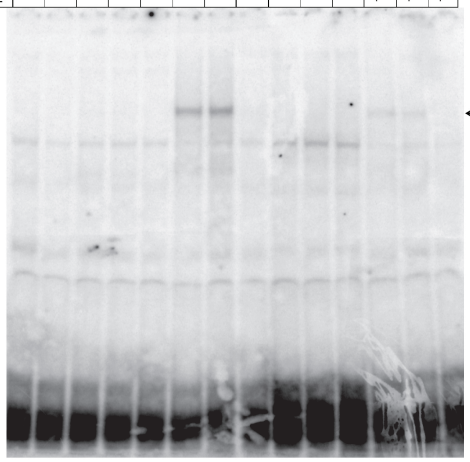
SUPPLEMENTARY FIGURE

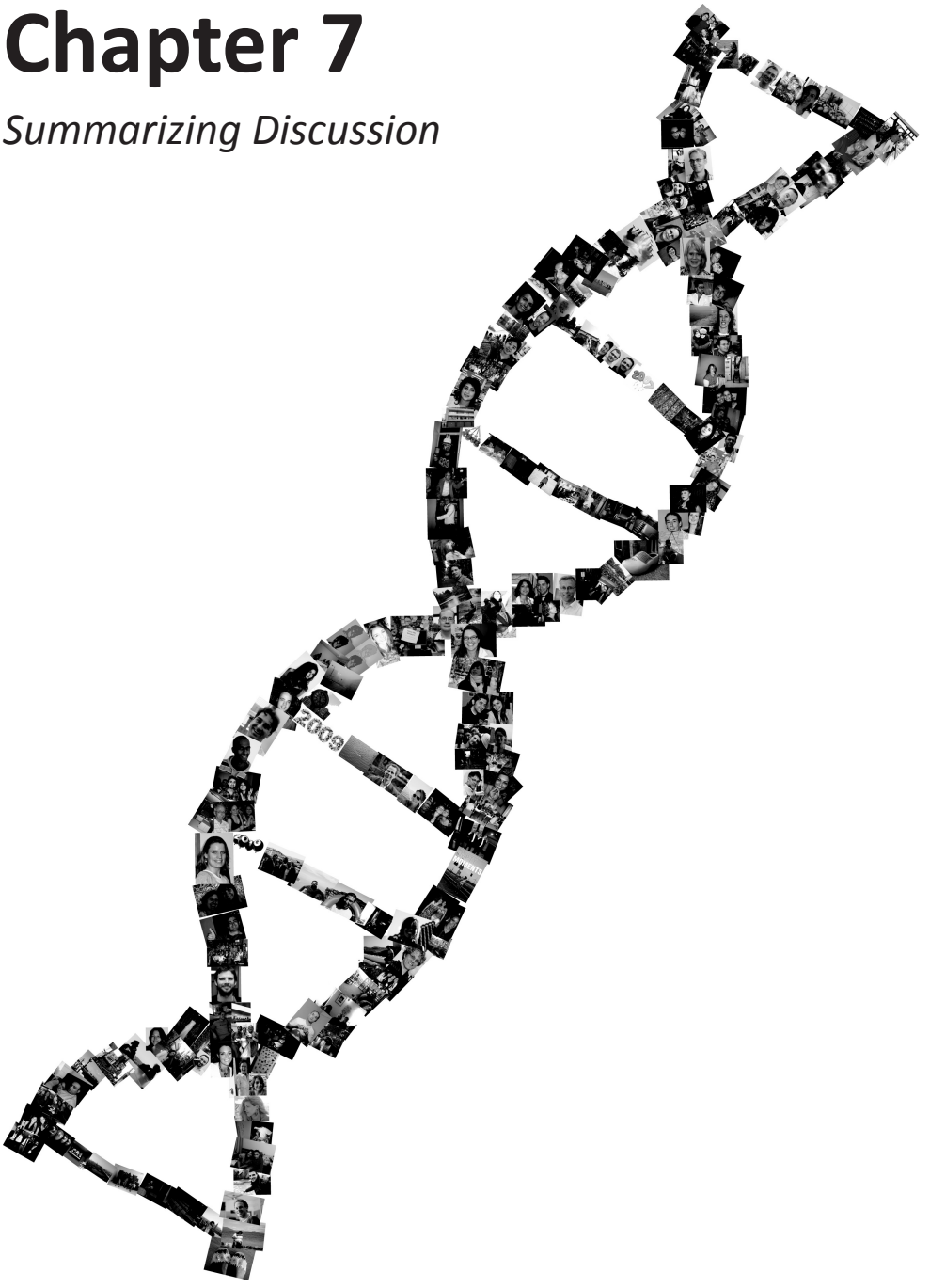
EV	+												
RXR		+				+	+	+	+	+	+	+	+
FXR WT			+			+			+			+	
FXR-S224D				+			+			+			+	
FXR-S224A					+			+			+			+
SHP FXRE	+	+	+	+	+	+	+	+				+	+	+
Mut SHP FXRE									+	+	+			
Excess cold SHP FXRE												+	+	+



Supplementary Figure 1: *FXR-S224A but not FXR-S224D is defective in DNA binding to FXREs.* EMSA using *in vitro* translated empty vector (EV), FXR-WT, FXR-S224D, FXR-S224A and RXR proteins and a radiolabeled or cold oligonucleotide corresponding to the IR-1 FXR responsive element from SHP promoters (WT or mutant), as indicated. The FXR/RXR heterodimeric complex is indicated.

Chapter 7

Summarizing Discussion



Inflammatory bowel disease (IBD) is a multifactorial intestinal disorder, characterized by chronic intestinal inflammation. Genetic susceptible hosts display a hyperactivation of the immune response against the enteric commensal flora and disruption of the intestinal epithelial barrier, together causing the onset or reactivation of this pathologic condition[1]. Bile salts are currently recognized as signalling molecules acting at various levels within the gastrointestinal tract. On the one hand, they exert their classical role as lipid solubilizers in the intestinal lumen, whereas on the other hand they exploit their function as ligands of the bile salt nuclear Farnesoid X Receptor (FXR)[2]. Bile salts are highly toxic, and it was discovered that FXR is the master regulator of bile salt concentrations in cells by regulating the inward and outward bile salt transport systems, bile salt metabolism and the *de novo* synthesis from cholesterol in the liver[3]. FXR modulates the expression of its target genes by heterodimerizing with the retinoid X receptor (RXR, NR2B1) to FXR responsive elements (FXREs) in the DNA. FXR not only regulates bile salt homeostasis, but is also implicated in liver regeneration[4], carcinogenesis[5-7], energy expenditure[8], as well as lipid[9] and glucose homeostasis[10] (reviewed in **Chapter 1**). In the research described in this thesis we aimed to further explore the role of FXR expression and activation in the context of intestinal inflammation. In the following paragraphs, I will address the research questions raised in the introduction.

Does FXR activation ameliorate intestinal inflammation? Does FXR activation suppress the NF-κB inflammatory pathway?

In **Chapter 2** we describe a novel role of FXR activation in inhibiting inflammation and preserving the intestinal barrier integrity in two models of murine colitis. Our findings revealed that the FXR semi-synthetic ligand INT-747[11] significantly decreased the severity of DSS- and TNBS-induced colitis in mice, as indicated by decreased body weight loss, colon shortening, and rectal bleeding, improvement of colonic histology as well as decrease of endothelial ulceration and partial prevention of mucin-secreting goblet cell loss. This improvement was not seen in Fxr-ko mice, underscoring that the amelioration of colitis by INT-747 specifically requires Fxr. Additionally, Fxr activation nearly normalizes DSS- and TNBS-induced colonic pro-inflammatory gene expression and markedly induces antibacterial defense gene expression in wild type (WT) but not in Fxr-ko mice. Furthermore, Fxr activation notably decreases DSS- and TNBS- induced intestinal permeability *in vivo* in WT mice and *in*

vitro in a Caco-2 transwell set-up, highlighting the role of FXR in preserving the integrity of the intestinal epithelial barrier. The observed inhibition of cytokine expression by FXR activation may represent a possible mechanism preserving intestinal permeability, since several cytokines (TNFα, IFNγ, IL-4 and IL-13)[12,13] are known to increase intestinal permeability in the intestinal epithelial monolayer by modulating tight junction protein expression and localization. Alternatively, FXR may have a direct effect on the integrity of the intestinal mucosa. This view is strongly supported by the fact that Fxr-ko mice display an increased intestinal permeability at baseline (Chapter 2, Supplementary Figure 5) with unaltered cytokine levels (Chapter 2, Figure 4). FXR is mainly expressed in differentiated enterocytes[14]. However, it is also present in immune cells[15]. Based on our results it is not clear whether the overall protection can be attributed to the enterocytes or immune cells, or whether they have a synergistic effect. We show *in vitro* that INT-747 protects against DSS-induced permeability of a monolayer of enterocyte-like Caco-2 cells and that TNFα-induced *IL-1β* mRNA expression is drastically reduced upon FXR activation in differentiated enterocyte-like HT29 cells. On the other hand, we show that INT-747 decreased the inflammatory response in primary human immune cell types, PBMCs, CD14+ monocytes and monocyte-derived dendritic cells isolated from healthy controls, and reduced inflammatory signalling in lamina propria mononuclear cells (LPMCs) isolated from IBD patients as well. It is generally accepted that intestinal epithelial cells (IECs) modulate the immune response, although the exact mechanism is still not clear[16-18]. *In vitro* studies have implicated a cross-talk between IECs and immune cells in mounting the immune response in the gastrointestinal tract[19-21]. Data from Zaph *and colleagues* show a more direct role of the IECs in influencing innate and adaptive immunity in the gut *in vivo*[22], demonstrating the crucial role of IECs in pathogen recognition and initiation of the immune response. To further underline the importance of IECs, mice with targeted disruption of the PPARγ gene in IECs display more severe DSS-induced colitis compared to their WT littermates, indicating that endogenous PPARγ in IECs has a major protective effect against intestinal inflammation[23]. However, the contribution of enterocytes and immune cells in FXR-mediated protection against intestinal inflammation needs to be studied in more detail. The precise molecular mechanisms by which FXR confers its anti-inflammatory effect are presently unclear. Vavassori *et al.* show that ligand-bound FXR is

recruited to the iNOs promoter and their data suggest that activated FXR inhibits nuclear corepressor (NCoR) clearance from NF-κB/DNA complex on NF-κB target gene promoters[24]. We show in **Chapter 3** (Figure 3) that FXR can bind the two most common NF-κB subunits, p50 and p65/RelA in GST-pull down assays and that FXR interferes with NF-κB transcriptional activity In addition, data from Wang *et al.* suggest that FXR activation suppressed NF-κB transcriptional activity by decreasing the binding between NF-κB and DNA sequences[25]. Whether these results can be combined in one singular mechanism or that there are multiple mechanisms by which FXR interferes with NF-κB mediated inflammatory signalling is a topic for further investigation. For other NRs interfering with inflammatory signalling, a multitude of mechanisms have been described, comprising DNA tethering, cofactor squelching and others (for more details, see Chapter 1, Figure 3)[26].

Is FXR activation impaired under intestinal inflammatory conditions?
In recent years, a reciprocal repression between several NRs and inflammatory pathways has been described. It is well known that activated NF-κB downregulates the transcriptional activity of many NRs, such as the Steroid and Xenobiotic/Pregnane X receptor (SXR/PXR)[27] and peroxisome-proliferator activating receptor gamma (PPARγ)[28]. Moreover, activation of Glucocorticoid Receptor (GR), Androgen Receptor (AR) or Estrogen Receptor (ER) inhibits inflammation, whereas activation of these nuclear receptors is highly reduced in inflamed tissues, suggesting that repression of NRs might be a mechanism required for inflammation to progress[29]. In **Chapter 3** and **4**, we show that FXR and NF-κB mutually repress each other as well. In **Chapter 3** we show that not only does FXR inhibit inflammation (as shown in Chapter 2), but also FXR activation is inhibited by pro-inflammatory stimuli in different model systems. *In vitro*, the pro-inflammatory cytokine TNFα decreases FXR target gene expression in differentiated enterocyte-like HT29 cells. Additionally, reporter assays show that the transactivation capacity of FXR on target genes IBABP and SHP promoters is abolished in the presence of the FXR synthetic ligand GW4064 and either TNFα or IL-1β or upon over-expression of the NF-κB subunits p50 and p65/RelA. The *in vitro* inhibition of FXR activity by inflammatory stimuli was confirmed in ileal specimens of WT mice cultured *ex vivo*. Moreover, in mice with severe intestinal inflammation induced by DSS[30], the expression of Fxr target genes *Ibabp* and *Fgf15* was similarly reduced. These results are in line with data from Wang *and*

colleagues, who reported that NF-κB activation suppresses FXR-mediated gene expression both *in vitro* and *in vivo* in hepatocytes[25]. Several possible mechanisms for different NRs have been described in this respect, such as the p65/RelA-dependent disruption of binding between PXR/RXR complex and its responsive DNA element[31]. We examined whether a similar mechanism exists for FXR. As described in the previous paragraph, in **Chapter 3** we show physical binding between the two NF-κB sub-units, p50 and p65/RelA and FXR in GST-pull down assays. However, preliminary data suggest that FXR-DNA binding is unaffected in the presence of p50 and RelA in EMSAs (data not shown). At this stage, DNA tethering cannot be excluded and other mechanisms may well be possible, such as the competition between NRs and the NF-κB complex for a common transcriptional cofactors pool ("cofactor squelching").

Is FXR activation repressed in IBD patients and are FXR polymorphisms associated with IBD?

The findings in Chapter 3 that FXR is repressed by inflammatory signals led us to investigate *FXR* and FXR target gene mRNA expression in IBD patients. In **Chapter 4**, we show that mRNA expression of *FXR* is unaltered in IBD patients compared to controls. This is in line with the results obtained in Chapter 3, in which we show that *FXR* expression does not change in the different models, upon TNFα, IL-1β and DSS administration. However, also in line with results in Chapter 3, mRNA expression of the FXR target gene *SHP* is significantly reduced by 50% in patients with Crohn's disease in clinical remission compared to controls. On the contrary, *SHP* mRNA expression in UC patients was not significantly decreased. These results suggest that FXR activation is specifically decreased in patients with Crohn's disease. All together, these studies underline that FXR is not only an active player in inhibition of inflammation, but also is a target of the inflammatory response itself. This could result in a vicious cycle where reduced FXR activity results in less repression of intestinal inflammation, contributing to development of chronic intestinal inflammation. Genetic predisposition is a key feature in IBD pathogenesis. Familial clustering of cases and twin studies have established a role for genetic factors and in the last decade several genes have been associated with IBD. Crucial genes in the pathogenesis of CD and/or UC are involved in the immune response (e.g. NOD2, IL12B, IL23R, STAT3, MHC, IL-10, IFNγ), authophagy (e.g. ATG16L1, IRGM), antibacterial defense (e.g. cathelicidin, defensins), as well as in barrier function (e.g. PTPsigma, MAGI2, myosin IXB and E-cadherin)[32-35].

Although the total number of susceptibility loci amounts to 90 in recent genome-wide association studies[36], this probably accounts for only 16% of UC heritability[36] and 20% of CD heritability[37] of the genetic contribution in IBD. Since FXR acts as a regulator of intestinal inflammation, we hypothesized that also mutations/functional polymorphisms in FXR might be associated with IBD. In **Chapter 4** we investigated this hypothesis by genotyping seven tagging SNPs and two functional polymorphisms in FXR in a cohort of 1162 CD patients, 1193 UC patients and 853 healthy controls. Some of the SNPs were weakly associated with IBD subtypes, however associations were not significant after Bonferroni correction for multiple testing, suggesting that the FXR gene is not a major IBD locus. Intriguingly, the 518t>c polymorphism, resulting in the FXR-M173T variant, was weakly associated with ileocolonic CD (p=0.015, OR 1.05, 95% CI 1.08-8.83). The FXR-M173T variant has previously been shown to be associated with intrahepatic cholestasis of pregnancy[38], and *in vitro* studies showed that this amino acid change results in a 60% decrease in transcriptional activity by FXR. Although the M173T did not significantly associate with Crohn's colitis after correction for multiple testing, we cannot exclude the possibility that it plays a modifier role in the etiology of Crohn's colitis in conjunction with other loci. In addition to reduced FXR activity caused by inflammatory signals, genetically reduced FXR activity due to the M173T polymorphism might influence the CD phenotype.

Is FXR expression and/or activation impaired in IBD patients with concurrent cholestatic liver disease?

From another perspective, FXR inhibition by inflammatory signals in the intestine can also cause disturbance in the enterohepatic circulation of bile salts. Indeed, it has been shown that CD patients have smaller gallbladders[39] and have an increased risk of developing gallstones[40]. Additionally, IBD patients display a faster intestinal transit times, due to increased diarrhea[41]. This might result in less bile salt absorption and consequently lower FXR activation. Moreover, an inflammation-related decrease in FXR activity is potentially relevant for cholestatic liver disease, which often coexists in patients with IBD. A significant proportion of IBD patients are known with primary sclerosing cholangitis (PSC). PSC is a chronic liver disease characterized by progressive inflammation that damages bile ducts both inside and outside the liver. The resulting scarring of the bile ducts blocks the flow of bile, causing cholestasis and ultimately leads to liver cirrhosis, liver failure and liver cancer. In **Chapter**

5 we hypothesized that PSC patients with IBD may also have decreased FXR activation in the intestine due to decreased bile salt excretion in the intestine. Unlike in CD patients (Chapter 4), there is no significant change in mRNA expression of *SHP* in patients with PSC and coexistent Crohn-like colitis. However, the significant correlation between the expression of the enterocyte-differentiation marker *Villin* and either *FXR* or *SHP* found in normal controls is lost in PSC patients with coexistent Crohn-like colitis, indicating that *FXR* and *SHP* expression as a percentage of *Villin* expression is reduced. In addition, we found a strongly increased proportion of primary bile salts at baseline as well as a relative hydrophilic bile salt pool in patients with IBD and PSC. The hydrophilicity of the bile salt pool was further increased during treatment with ursodeoxycholic acid (with approximately 30% enrichment with UDCA). Since UDCA is a poor ligand for FXR, a decrease in FXR activity and concurrent FXR target gene expression in the enterocyte could be anticipated. Phosphatidylcholine molecular species composition may strongly affect behavior of bile salts in mixed micellar solutions. However, this was not clearly different from previously reported control groups nor affected by ursodeoxycholic acid therapy.

What are the molecular mechanisms underlying FXR transactivation and transrepression?

The classical mechanism by which NRs regulate gene transcription is via transactivation, which requires ligand binding, direct binding of the NR to NR-response elements, with consequent release of co-repressor and recruitment of co-activator complexes. On the contrary, the mechanism used by NRs to inhibit the inflammatory response is generally via transrepression (Figure 1). Co-regulators and post-translational modifications are ultimate components of NR signalling which dictate these two different NR functions[42]. Recently it has been shown that FXR can be phosphorylated[43], acetylated[44] and sumoylated[24]. However, it is not known whether these modifications are specifically important for transactivation, transrepression or both. In **Chapter 6**, we aimed to start dissecting the molecular pathways regulating FXR transactivation and transrepression. We describe a new phosphorylation site in FXR (FXRα2-S224), identified with an unbiased mass spectrometry approach. Substitution of Serine 224 with Alanine revealed that this site is essential for transactivation, but not for transrepression of NF-κB signalling. We show that this is due to the inability of FXR-S224A to bind FXR responsive

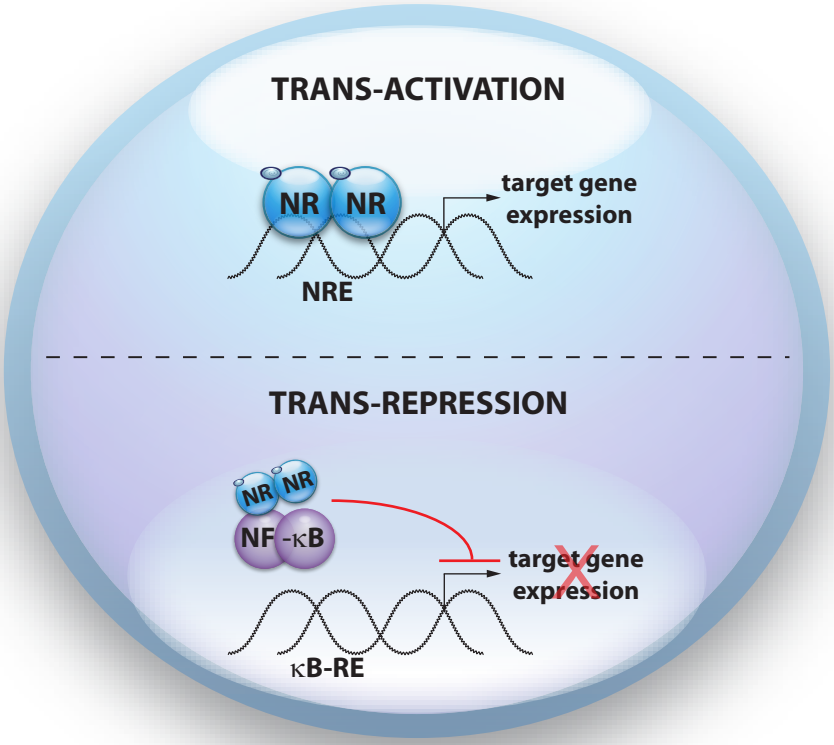


Figure 1: *Schematic representation of the 2 different transcriptional actions of NRs* (see text for details; NRE: Nuclear receptor response element; κB-RE: κB response element).

elements. Although many post-translational modifications have been identified within the NR superfamily, to our knowledge, this is the first finding of a post-translational modification in an NR that is differentially regulating transactivation and transrepression. These findings potentially advance drug design for FXR, since selective FXR ligands which do not result in S224 phosphorylation may be useful to treat hepatic/intestinal inflammation without interefering with bile salt, glucose and fat metabolism (Figure 2).

Is FXR a valuable target for the treatment of intestinal inflammation?
A significant proportion of IBD patients are initially treated with

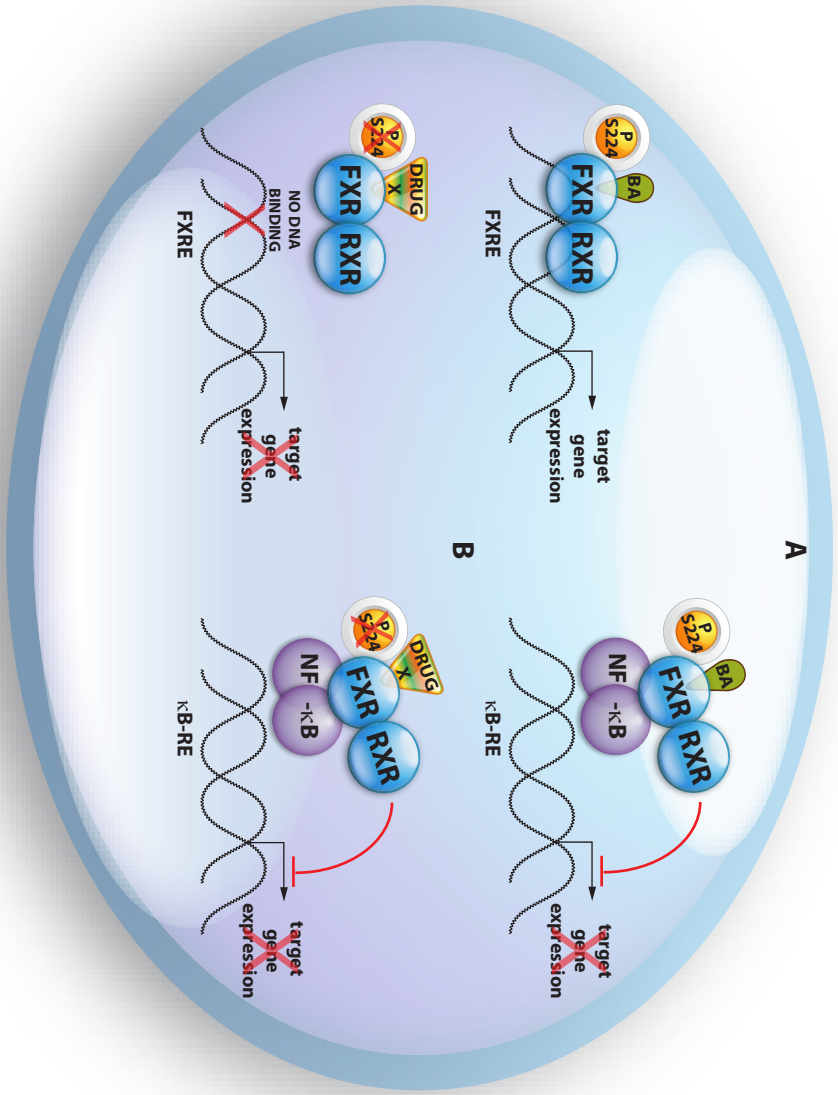


Figure 2: *Hypothetical model of selective FXR ligands dissociating transactivation and transrepression, based on differential phosphorylation of S224.*

(A) Full FXR agonist will trigger transactivation of FXR target gene as well as trans-repression of NF-κB target genes.
(B) Hypothetical drug X prohibits phosphorylation of FXR-S224, preventing FXR from binding to FXREs and exclusively allowing transrepression of NF-κB target genes (see text for details).

corticosteroids, targeting the glucocorticoid receptor. This approach is usually effective in controlling the symptoms, however many patients become resistant and long exposure to these drugs is frequently accompanied by several severe side effects (such as osteoporosis, insulin resistance, dyslipidemia, and others). This is generally thought to result from the general activation of all transcriptional actions of the NR (transactivation and transrepression). Biological therapies targeting TNFα, such as Infliximab, have improved the management of refractory CD patients, although loss of response and side effects, such as infections, are frequently encountered. There is therefore a clear need for alternative therapies. Because of the large variation in patients responsiveness and the severe side effects of the classical therapies, the present direction for the clinical management of IBD aim towards customized treatment to meet each individual patient's needs. Combination therapies targeting NRs, such as corticosteroids in combination with PPARα agonists are under investigation, trying to gain the advantage of lowering the single dosages with synergistic effectiveness[45,46]. The results described in this thesis indicate that FXR is an important player in the counter-regulation of intestinal inflammation. Although the exact mechanism is still unclear, there are strong indications that modulation of FXR expression/activity may be transferred from bench to bedside, by using it as a drug target for intestinal inflammatory disorders. INT-747/6-ECDCA is a potent FXR agonist and we show that INT-747 administration protects against intestinal inflammation in murine colitis. INT-747 is currently in phase 2 and phase 3 trials in patients with type 2 diabetes with fatty liver disease and in patients with primary biliary cirrhosis, demonstrating its safety and patient's tolerance to this drug. Caution should be taken to extend these trials to patients with IBD, because chronic treatment with INT-747 may induce side effects, due to parallel FXR-dependent transactivation and transrepression of gene transcription. Dissociated NR agonists altering the balance between transactivation and transrepression mechanisms are currently topic of intense research, in particular for the GR[47,48]. The challenge for the immediate future will therefore be to pursue the functional dissection

of FXR transcriptional functions (transactivation and transrepression). This will advance NR-targeted drug design in general and FXR-targeted drug design specifically, and will eminently lead to the development of a second generation of NR-targeted drugs with fewer side effects for IBD patients.

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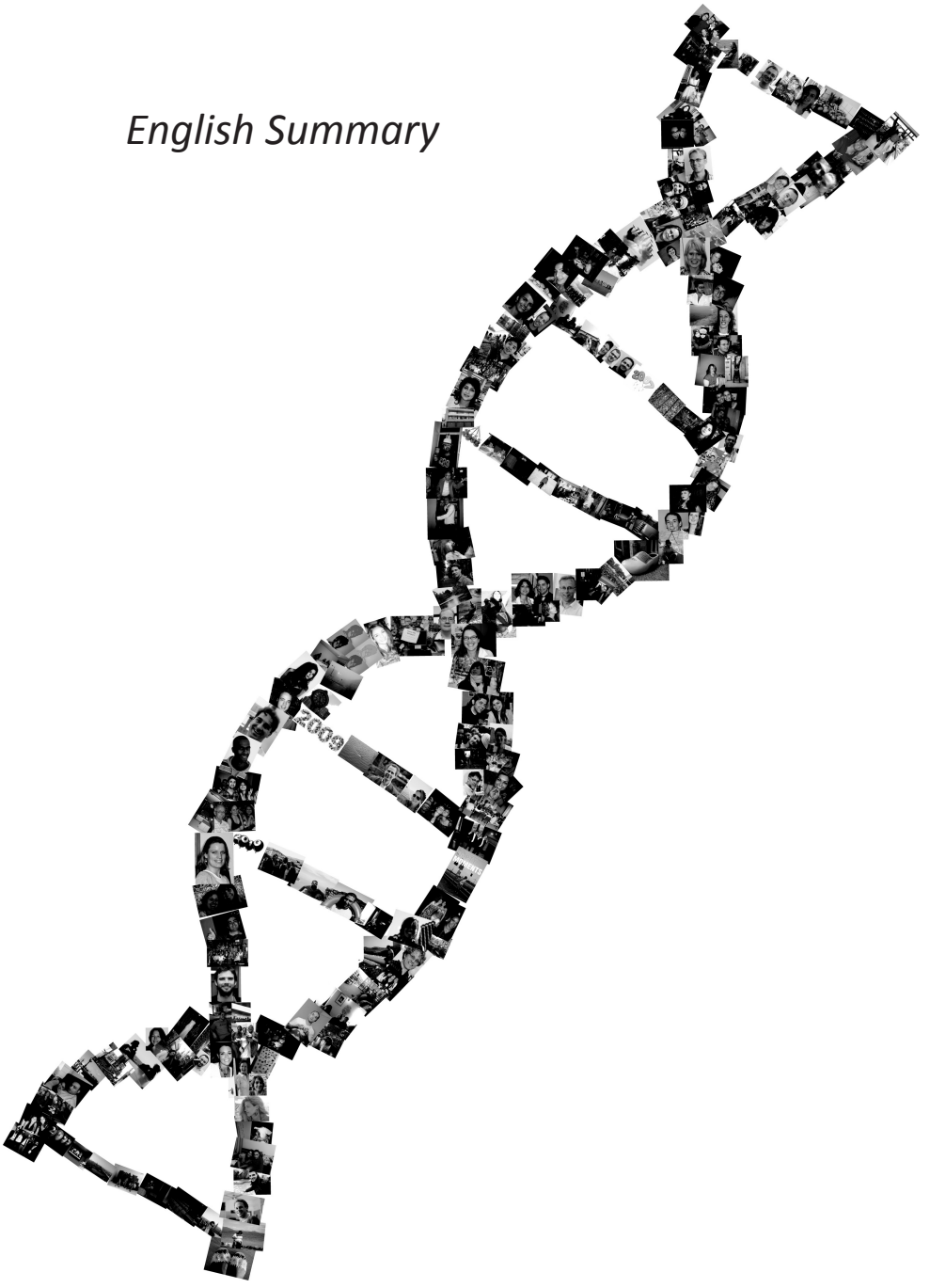
English Summary



Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory disorder, characterized by dysregulation of the mucosal immune system and compromised intestinal epithelial barrier. In both phenotypes of IBD, Crohn's disease (CD) and ulcerative colitis (UC), the immunological balance between pro-inflammatory and anti-inflammatory mediators is severely impaired and shifted towards the pro-inflammatory side. The nuclear transcription factor kappa B (NF-κB) was identified as one of the key regulators in this shift. The bile salt nuclear Farnesoid X Receptor (FXR) is a member of the nuclear receptor (NR) superfamily. Nuclear receptors are ligand-activated transcription factors that, in response to lipophilic ligands (e.g. hormones, vitamins and dietary lipids), regulate many aspects of mammalian physiology, including development, reproduction and metabolism. FXR is mainly expressed in the liver and gastrointestinal tract and it is activated by endogenous bile salts and regulates transcription of genes involved in bile salt synthesis, transport and metabolism. FXR not only regulates bile salt homeostasis, but is also implicated in liver regeneration, carcinogenesis, energy expenditure, as well as lipid and glucose homeostasis. In recent years, functional cross-talk between NF-κB and several NRs has been demonstrated. NRs were shown to have anti-inflammatory properties, by interfering with NF-κB function. On the other hand, activated NF-κB is described to downregulate the transcriptional activity of several NRs. Indeed, activation of several NRs inhibits inflammation, whereas activation of these nuclear receptors is highly reduced in inflamed tissues. This suggests that repression of NRs might be a mechanism required for inflammation to progress.

In this thesis, we aimed to explore the role of FXR in maintaining a healthy gut and the potential therapeutic implications of FXR pharmacological activation for inflammatory bowel diseases.

In **Chapter 1**, we review the current status of the literature on the relevance of FXR activity in hepatobiliary and intestinal disease. In the subsequent chapters, we address the following research questions:

Does FXR activation ameliorate intestinal inflammation? Does FXR activation suppress the NF-κB inflammatory pathway?
In **Chapter 2**, we describe a novel role of FXR activation in inhibiting

inflammation and preserving the intestinal barrier integrity. Pharmacological FXR activation, with the semi-synthetic FXR ligand 6-EthylChenodeoxycholic (6-ECDCA/INT-747), resulted in a decrease in the severity of inflammation and preserved the intestinal barrier integrity in two well-established murine colitis models. In particular, Fxr activation improved colitis symptoms, protected from intestinal mucin-secreting goblet cells loss and preserved the integrity of the intestinal epithelial barrier. Moreover, pharmacological FXR activation decreased mRNA expression of several pro-inflammatory genes (such as *Il-1β*) and increases the mRNA expression of antibacterial defense genes (*cathelicidin*, *iNOs*, *Ang1*), both *in vitro* and *in vivo*. Additionally, we showed that FXR activation inhibits inflammatory signalling in various primary human immune cell types (PBMCs, CD14+ monocytes and monocyte-derived dendritic cells) and the anti-inflammatory properties of FXR activation are confirmed ex vivo in lamina propria mononuclear cells (LPMCs) from IBD patients. The precise molecular mechanisms by which FXR confers its anti-inflammatory effect are presently unclear. However, the multilevel protection against intestinal inflammation provides a clear rationale to further explore FXR agonists as a novel therapeutic strategy for IBD.

Is FXR activation impaired under intestinal inflammatory conditions?
In **Chapter 3**, we examined whether there is mutual antagonism between NF-κB and FXR, as has been reported for other nuclear receptors. By complementary *in vitro*, *ex vivo* and *in vivo* approaches, we show that FXR and NF-κB mutually repress each other. *In vitro*, the pro-inflammatory cytokine TNFα decreases FXR target gene expression in differentiated enterocyte-like HT29 cells. Additionally, reporter assays show that the transactivation capacity of FXR on target genes IBABP and SHP promoters is abolished in the presence of the FXR synthetic ligand GW4064 and either TNFα or IL-1β or upon over-expression of the NF-κB subunits p50 and p65. The *in vitro* inhibition of FXR activity by inflammatory stimuli was confirmed in ileal specimens of WT mice cultured *ex vivo*. Moreover, in mice with severe intestinal inflammation induced by DSS, the expression of Fxr target genes *Ibabp* and *Fgf15* was similarly reduced. Several possible mechanisms for different NRs have been described in this respect, such as the p65-dependent disruption of binding between NR complex and its responsive DNA element. In Chapter 3, physical binding between the two NF-κB sub-units, p50 and p65 and FXR is shown in

GST-pull down assays. However, other mechanisms cannot be excluded. In this chapter we show that FXR is not only an active player in inhibition of inflammation, but is a target of the inflammatory response itself. This could result in a vicious circle where reduced FXR activity results in less repression of intestinal inflammation, contributing to the development of chronic intestinal inflammation. The reciprocal antagonism between NF-κB and FXR indicates an interaction between lipid metabolism and inflammatory diseases and leads to new insights in pathogenesis and to novel treatment strategies for IBD.

Is FXR activation repressed in IBD patients and are FXR polymorphisms associated with IBD?

In **Chapter 4**, we analyzed *FXR* and FXR target gene *SHP* expression in ileal and colonic biopsies of IBD patients. In line with results in Chapter 3, we show that mRNA expression of the FXR target gene *SHP* is significantly reduced by 50% in patients with CD in clinical remission compared to controls. On the contrary, *SHP* mRNA expression in UC patients was not significantly decreased. We hypothesized that genetic variation in FXR may confer susceptibility to subgroups of CD and UC colitis and we therefore evaluated whether tagging and functional polymorphisms in the FXR gene were present in a cohort of 2355 IBD patients and 853 healthy controls. None of the tagging SNPs was significantly associated with UC or CD. Although the polymorphism 518t>c (M173T, leading to a 60% decrease in FXR protein function), showed an association with the ileocolonic phenotype of CD, this and other weak associations of different tagging SNPs with ileocolonic or colonic phenotypes disappeared after correction for multiple testing. Together, these data indicated that FXR activity is decreased in IBD patients although at presence this cannot be explained by genetic variation in the FXR gene. Treatment with synthetic FXR agonists may overcome the decrease in FXR activation, possibly resulting in an amelioration of ileocolitis in patients with CD.

Is FXR expression and/or activation impaired in IBD patients with concurrent liver disease?

From another perspective, FXR inhibition by inflammatory signals in the intestine can also cause disturbance in the enterohepatic circulation of bile salts. Indeed, a significant proportion of IBD patients are suffering from primary sclerosing cholangitis (PSC). In **Chapter 5** we determined bile salt

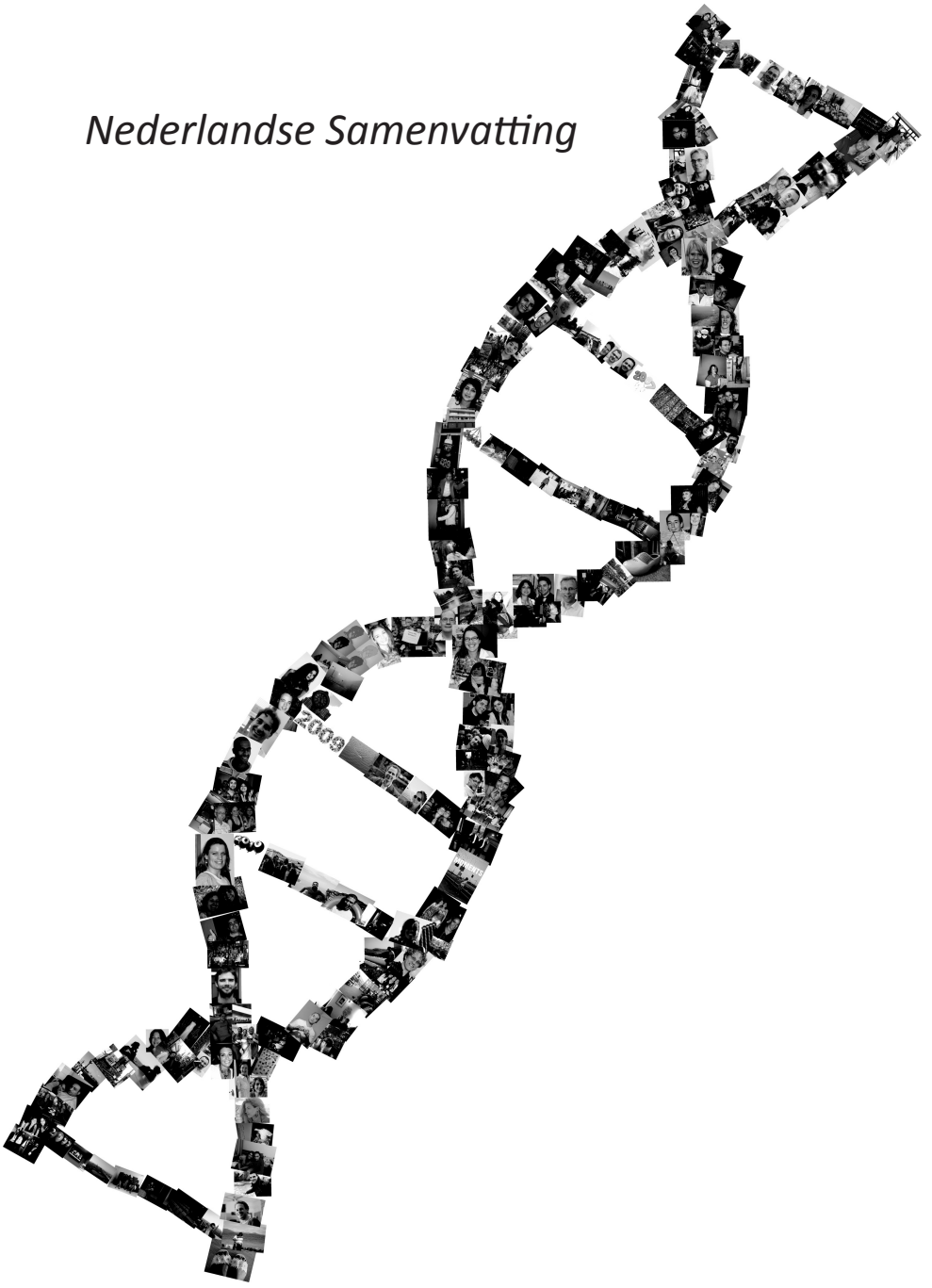
and phospholipid molecular species composition by HPLC in duodenal bile of IBD-PSC patients and mRNA expression levels of *FXR* and its target gene *SHP* were determined in ileal biopsies of additional 15 IBD-PSC patients (all patients were on treatment with UDCA). There were no significant differences in ileal *FXR* and *SHP* expression between controls and PSC patients with Crohn-like colitis or UC, despite hydrophilic bile salt pool enriched in UDCA. Potential therapeutic effects of the currently available powerful FXR agonists in this patient category remain to be explored.

What are the molecular mechanisms underlying FXR transactivation and transrepression?
The classical mechanism by which FXR regulates gene transcription is via transactivation, which requires ligand binding, direct binding of the FXR to FXR response elements, with consequent release of co-repressor and recruitment of co-activator complexes. On the contrary, in chapter 2, we provide evidence for a novel role in transrepression of NF-κB signaling. In **Chapter 6**, we start dissecting the differential mechanisms for classical transactivation and transrepression of FXR, by identification and characterization of post-translational modifications (PTMs) in FXR. We identified a new phosphorylation site in FXR (FXRα2-S224). Substitution of Serine 224 with Alanine revealed that this site is essential for transactivation, but not for transrepression of NF-κB signalling. We show that this is due to the inability of FXR-S224A to bind FXR responsive elements. These findings potentially advance drug design for FXR, since selective FXR ligands which do not result in S224 phosphorylation may be useful to treat hepatic/intestinal inflammation without interfering with bile salt, glucose and fat metabolism.

Is FXR a valuable target for the treatment of intestinal inflammation?
In the summarizing discussion (**Chapter 7**) the classical role of FXR controlling bile salt homeostasis and the new role of FXR in intestinal inflammation are discussed. A significant proportion of IBD patients are initially treated with immune suppressants, however this approach is often accompanied by long term-related side effects. This is generally thought to result from the general activation of all transcriptional actions of the NR (transactivation and transrepression). The results described in this thesis indicate that FXR is an important player in the counter-regulation of intestinal inflammation.

Although the exact mechanism is still unclear, there are strong indications that modulation of FXR expression/activity may be transferred from bench to bedside, by using it as a drug target for intestinal inflammatory disorders.

Nederlandse Samenvatting



Chronische inflammatoire darmziekte (in het Engels: inflammatory bowel disease, IBD) is een ontstekingsziekte van het darmkanaal, die wordt gekarakteriseerd door ontregeling van het mucosale immuun systeem en gecompromitteerde epitheliale integriteit. Chronische inflammatoire darmziekte is in te delen in twee fenotypes, de ziekte van Crohn (in het Engels Crohns Disease; CD) en colitis ulcerosa (in het Engels Ulcerative Colitis; UC). In beide fenotypes is de immunologische balans tussen pro-inflammatoire en anti-inflammatoire mediatoren verschoven naar een pro-inflammatoire respons. Er is beschreven dat de nucleaire transcriptie factor kappa B (NF-κB) zeer belangrijk is in deze balansverschuiving. Nucleaire receptoren (NR's) zijn ligand-geactiveerde transcriptie factoren die na binding van specifieke liganden (bv. hormonen, vitamines en vetten uit het dieet) vele aspecten van normale fysiologie in het lichaam reguleren, zoals ontwikkeling, voortplanting en metabolisme. De nucleaire receptor die galzout homeostase reguleert is de Farnesoid X Receptor (FXR). FXR komt name tot expressie in de lever en in de darm en wordt geactiveerd door endogene galzouten. Geactiveerd FXR reguleert de transcriptie van genen betrokken bij synthese, transport en metabolisme van galzouten. Naast galzout homeostase, speelt FXR ook een rol in lever regeneratie, tumorontwikkeling, energie huishouding en vetstof- en glucosehomeostase. Recent is aangetoond dat er functionele wederkerige relaties bestaan tussen NF-κB en verschillende andere NRs. Deze NRs bezitten anti-inflammatiore capaciteiten, door de NF-κB functie te remmen. Aan de andere kant is ook beschreven dat NF-κB de functie van deze andere NRs remt. Inderdaad leidt activatie van verschillende NRs tot verminderde ontsteking, en is activatie van NRs in ontstoken weefsels geremd. Dit suggereert dat repressie van NRs mogelijk een mechanisme is voor het ontstaan en het uitbreiden van ontstekingen.

Het doel van het onderzoek beschreven in dit proefschrift is om de rol van FXR bij het in stand houden van een gezonde darm te onderzoeken, evenals de mogelijke therapeutische implicaties van farmacologische FXR activatie voor chronische inflammatoire darmziekten.

In **Hoofdstuk 1**, wordt de huidige literatuur betreffende de relevantie van FXR activatie in hepatobiliaire en darmziekten uiteen gezet. In de daaropvolgende hoofdstukken worden de volgende onderzoeksvragen onderzocht:

Wordt darmontsteking geremd door activatie van FXR? En wordt de NF-κB signaalroute geremd door activatie van FXR?
In **Hoofdstuk 2** beschrijven we een nieuwe functie van FXR in het remmen van ontstekingen en het behoud van de intestinale epitheliale integriteit. Farmacologische activatie met het semisynthetische ligand 6-ethyl-chenodeoxycholic acid (6-ECDCA/INT-747), resulteert in verminderde ontsteking en verminderde permeabiliteit in twee goed bestudeerde darmontstekingsmodellen in muizen. Meer specifiek, FXR activatie leidde tot verminderde mRNA expressie van verschillende pro-inflammatoire genen (zoals *Il-1β*) en toegenomen mRNA expressie van genen betrokken bij anti-bacteriele afweer (zoals *cathelicidin*, *iNOs*, *Ang1*), zowel *in vitro* als *in vivo*. Ook laten we zien dat FXR activatie inflammatoire signalering remt in diverse primaire immuuncel subpopulaties (PBMCs, CD14+ monocyten en dendritische cellen). Daarnaast worden de anti-inflammatoire eigenschappen van FXR nog eens ex vivo aangetoond in lamina propria mononucleaire cellen (LPMCs) van IBD patiënten. De precieze moleculaire mechanismen waarmee FXR darmontsteking remt worden in dit hoofdstuk niet opgehelderd. Echter, onze bevinding dat FXR activatie leidt tot verminderde darmontsteking geeft het zoeken naar alternatieve therapeutische strategieën voor IBD een nieuwe impuls.

Wordt FXR activiteit geremd door ontsteking gemedieerde signaleringsroutes?
In **Hoofdstuk 3** onderzochten we of er wederkerige communicatie bestaat tussen NF-κB en FXR, zoals voor andere NRs eerder is beschreven. Door middel van complementaire *in vitro*, *ex vivo* en *in vitro* experimenten, laten we zien dat FXR en NF-κB elkaar remmen. De pro-inflammatoire cytokine TNFα remt FXR targetgen expressie in vitro in gedifferentieerde, op enterocyte gelijkende HT29 cellen. Daarnaast laten we zien dat FXR activiteit zeer sterk geremd wordt in reporter assays wanneer de cellen met TNFα of IL-1β worden gestimuleerd of wanneer de NF-κB subunits p50 of p65 tot overexpressie worden gebracht. Ook ex vivo gekweekte darm specimen van wildtype muizen laten verminderde FXR targetgen expressie zien, wanneer deze worden geïncubeerd met TNFα of IL-1β. Tenslotte laten we zien dat in muizen met ernstige colitis, geïnduceerd door DSS (dextraan sodium sulfaat), FXR targetgen expressie is verminderd. Er zijn een aantal mechanismen beschreven die de repressie van NF-κB op NRs mogelijk kunnen verklaren, bijvoorbeeld dat NF-κB DNA binding van de nucleaire

receptor aan zijn responsieve element zou voorkomen. In hoofdstuk 3 laten we zien dat p50 en p65 interacties kunnen aangaan met FXR in GST pull down assays, maar op dit moment is het precieze mechanisme van de interacties nog niet opgehelderd. Samengevat laten we dus zien dat FXR niet alleen actief kan ingrijpen tegen ontsteking, maar ook door het ontstekingsproces zelf geremd wordt. Dit zou tot een vicieuze cirkel kunnen leiden waarin verminderde FXR activiteit resulteert in verminderde repressie van darm ontsteking, dat kan bijdragen aan de ontwikkeling van chronische darmontsteking. Dit mechanisme van wederkerig antagonisme tussen NF-κB en FXR laat zien dat er een interactie is tussen lipid metabolisme en ontstekingssignalering en kan leiden tot nieuwe inzichten in de pathogenese van IBD en tot nieuwe therapeutische strategieën voor IBD patiënten.

Wordt FXR activatie geremd in IBD patiënten en zijn FXR polymorfismen geassocieerd met IBD?

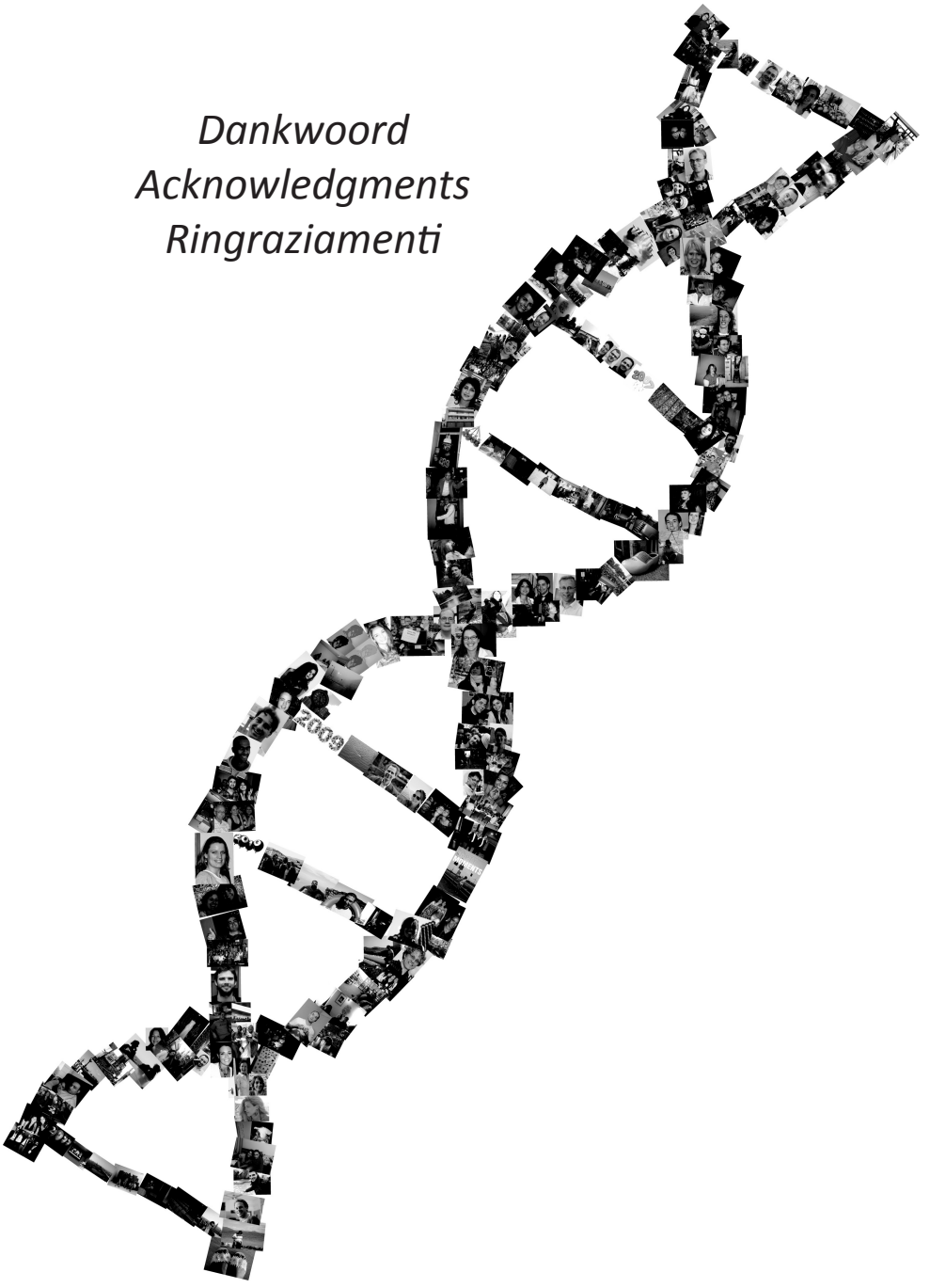
In **Hoofdstuk 4**, onderzochten we FXR en FXR targetgen expressie in dunne en dikke darm biopten van IBD patiënten. Vergelijkbaar met wat we beschreven hebben in hoofdstuk 3 in de muizendarm, laten we zien dat mRNA expressie van het FXR targetgen *SHP* significant verlaagd is in patiënten met de ziekte van Crohn die klinisch in remissie zijn. Dit in tegenstelling tot colitis ulcerosa patiënten, die geen vermindere SHP expressie lieten zien. We onderzochten de hypothese dat genetische variatie in het FXR-gen ten grondslag zou kunnen liggen aan subgroepen van IBD patiënten. Dit hebben we getest door middel van genetische analyse van het FXR-gen voor 7 tagging SNPs en 2 functionele polymorfismen in een cohort van 2355 IBD patiënten en 853 gezonde controles. Geen van de tagging SNPs was significant geassocieerd met UC of CD. Hoewel het polymorfisme 518t>c (M173T, leidend tot 60% afname in activiteit van FXR) een associatie liet zien met een ileocolonisch fenotype in CD patiënten, bleken deze en andere zwakke associaties niet significant na correctie voor meervoudige statistische toetsen. Samengevat laten we zien dat FXR activiteit is geremd in CD patiënten, maar dit kan niet verklaard worden door genetische variatie in FXR. Behandeling van IBD patiënten met synthetische FXR agonisten kunnen potentieel de remming van FXR activiteit tegen gaan, wat mogelijk resulteert in een verbetering van de ileocolitis in patiënten met CD.

Wordt FXR expressie en/of activatie geremd in IBD patiënten met lever problemen?
Remming van FXR functie door onsteking-gemedieerde signaalcascades zou ook van invloed kunnen zijn op de enterohepatische circulatie van galzouten. Inderdaad, hebben een significant aantal IBD patiënten ook primaire sclerosing cholangitis (PSC). In **Hoofdstuk 5** bepaalden we de galzout en fosfolipiden samenstelling in gal uit het duodenum van IBD-PSC patiënten. Daarnaast onderzochten we mRNA expressie van *FXR* en FXR targetgenen in darm biopten van deze patiënten. We laten zien dat er geen significante verschillen zijn in mRNA expressie van *FXR* en FXR targetgenen tussen controles en PSC patiënten met UC of CD, ondanks dat deze patiënten een zeer hydrofiele galzout samenstelling hebben verrijkt met UDCA. Potentiële therapeutische effecten van krachtige FXR agonisten bij deze patiëntengroep moeten verder worden onderzocht.

Wat zijn de moleculaire mechanismen waarmee FXR transactivatie en transrepressie worden gereguleerd?
Het klassieke mechanisme waarmee FXR gentranscriptie reguleert is via transactivatie van FXR. Na binding van het ligand aan FXR, bindt FXR direct aan specifieke responsieve elementen in promotoren van targetgenen, dit heeft tot gevolg dat corepressoren dissociëren en coactivatoren worden gerecruteerd. Nu beschrijven we in hoofdstuk 2 een tweede mechanisme waarop FXR genexpressie kan regularen; via transrepressie van NF-κB signalering. In **Hoofdstuk 6**, beginnen we met het ontrafelen van de differentiële mechanismen van transactivatie en transrepressie van FXR, door de identificatie en karakterisatie van post-translationele modificaties (PTM) in FXR. We hebben een nieuwe fosforylatie site in FXR geïdentificeerd (FXRα2-S224). Vervanging van Serine 224 door Alanine, laat zien dat deze fosforylatie belangrijk is voor transactivatie, maar niet voor transrepressie van NF-κB signalering. We laten zien dat FXRα2-S224 niet in staat is om aan DNA-response elementen te binden. Met deze bevindingen wordt het misschien mogelijk een nieuwe therapeutische strategie tegen chronische ontstekingen te genereren, omdat selectieve FXR liganden die niet in fosforylatie van S224 resulteren gebruikt kunnen worden om lever en darm ontstekingen te behandelen zonder te interfereren met galzout, glucose en vetstof metabolisme.

Is FXR een waardevolle target voor behandeling van darmontsteking?
In **Hoofdstuk 7** bediscussiëren we de klassieke rol van FXR in het reguleren van galzout homeostase en de nieuwe rol van FXR in darmontsteking. Een significant deel van de IBD patiënten worden initieel behandeld met immuun onderdrukkende medicijnen, hoewel deze behandelingsstrategie vaak samengaat met resistentie en bijwerkingen. Een groot deel van de bijwerkingen wordt toegeschreven aan generieke activatie van alle transcriptionele functies van een aantal nucleaire receptoren (transactivatie en transrepressie). De resultaten in dit proefschrift laten zien dat FXR een belangrijke speler is in de remming van darmontstekingen. Ondanks dat het mechanisme nog niet is opgehelderd, hebben we goede indicaties dat modulatie van FXR activiteit door synthetisch agonisten mogelijk een goed alternatief kan zijn voor behandeling van IBD patiënten.

Dankwoord
Acknowledgments
Ringraziamenti



This thesis would have not been possible without the support, the laughs, the gossip, the biertje, the tears, the smiles, the stamppotje, the lovely weather, the tulips and the virtual hugs I have got every day from all of you (and the people I'm sure I forgot to mention)…thanks!

Beste Professor Siersema, beste Peter, thank you very much for all your support and your enthusiasm for my project. Your presence has been instrumental for the success of my PhD.

Beste Professor Berger, beste Ruud, your presence has been subtle and discreet but in crucial moments you were there for me. I will never forget it.

Beste Karel, thank you very much for the mentorship, guidance and support you provided me all these years. You gave me the opportunity to combine my love for science and passion for medicine. You also taught me that criticism is constructive and an open mind is essential for learning. I hope you will soon have another Italian PhD student to continue this Bari-Utrecht axis in the best way!!! Hartelijk superbedankt!!!

Beste lieve Saskia, without you it would have not been the same! I deeply thank you for your guidance, patience and support. Your trust and belief in me crucially contributed to my development and kept me sane through this rollercoaster experience. We shared so much these years, ideas, results, technical failures, two pregnancies, flights, Croatia, Alex, bed, jokes and gossips. I wish you the best of luck with the new bigger FXR group. You have done amazingly well having a lovely family and being a PI. I truly hope we'll meet again for good science and great time!

Caro Antonio, non so dire a parole quanto ti ringrazio per avermi dato l'opportunità di far parte della tua scientific family, sia in Italia che in Olanda! Quando ho lasciato il Negri per l'Olanda ancora non sapevo quanto tutto questo mi avrebbe fatto crescere umanamente e scientificamente, e lo devo a te! Grazie, perchè anche a distanza mi sei sempre stato vicino.

Beste Bas, thank you for being so supportive in your amazing calm manner. I will never forget your super-supervising skills…always with a smile!

Beste Leo thank you very much for the uncountable number of useful discussions and the support you always offered me. Also, I will never forget not letting me alone in that canoe!!!

Mijn lieve paranymph Lieke, without you it would have not been the same! Gossip, cigarettes, borrels, the aluminium foil, everything for us was a reason to spice up our days!!! Thanks for everything...I am going to miss you a lot!

Lieve lieve Ellen you have been a crucial figure these past four years. Thank you for everything, for our happy dance, for the cloning, for inviting me to ride your bike in the only hilly place in the Netherlands and for your endless enthusiasm and contribution in our projects. FXR rocks!

Dear Stan, Stanny boy, Stanny-electric (or Geooooorge) thank you for all the support, advices and jokes, as a special bile-ologist, a roommate (and an Oscar nominee). Good luck with everything and I hope the zebrafish will make all your dreams come true!!!

Draga Angellus, one of the major contributors of the philosopher's wall, thank you very much for being a friend who I could have a laugh and an argument with. And this means a lot to me! Good luck with everything!

Dear colleague Henky, thank you very much for leaving my hair dry after I went to the hairdresser, in Milan. I really appreciated it! I enjoyed very much mixing my diplomatic skills with you straightforward ways. We should be an example to people trying to solve international conflicts. I wish you all the best!

Sevgili Ismy thank you very much for the trust you showed me and the jokes we shared during the past "Dr-wanna-be" years. You'll get there sooner than you expect. Thanks also for inviting me to your big big turkish wedding and teaching me the traditional turkish dances. I wish you all the best!

Qīnài de promovenda Yuan, you are almost there! Good luck with everything… and one day we'll meet again in Italy or China for some cultural enrichment.

Lieve Williane, with you I shared an artistic talent. I hope one day we'll share a stage too. I am going to miss your cheerful smile. I wish you all the best for your next adventure in the USA.

Lieve Monique, I enjoyed a lot our chatting. It has always been very pleasant to run into you on the bus. You are a warm and lovely person. I wish you all the best for the future.

Beste Patricia, Peter and Prim (equally contributed, in alphabetical order) it is a pity

that you have mostly experienced my somewhat pessimistic perspective on life. You helped me a lot to set up my new life in The Netherlands. Your sense of humor, your outgoing characters, your passion for Manolo Blahnik shoes (Patricia), your amazing cooking skills (Peter) and the dutch macho-latino resemblance (Prim), have been key for my future more cheerful development. I hope you are all having an amazing time and alles is goed! I hope we keep in touch.

Beste ex-colleagues Gemma, Leyla, Wendy, Annamieke and Bart thank you very much for the good times in-science and out-science. Hope you are all having a super-lekker tijd!

Agapiti Sophia, you are my favourite "fashion victim"! Your saying "I like pink but I am not pink" made it to my philosopher's wall! We only know each other for a few months, but having another mediterranean heart in the department has been really nice!

Beste colleagues Inkie, Mariette, Mateusz, Stiaan and Wendy, good luck with your projects. When you think that you've got a lot of PhD-S.H.I.T. (Special High Intensity Training), do not worry… there is much more on the way. But in the end you'll find that it was all worth it!

Beste Arjen, Wouter, Edwin, Rogier, Annamarie, Daniel, Gerdine, Marjiam, Janneke and Ingrid thanks for the time we spent either in our afdeling meeting, in the corridor, or at the "Basket" with a birtije and bitterballen!

Alex, agapi mou, dear Tzatziki! It is a pity that for a reason or another we do not succeed in spending more than three months in the same country! But you know, Felicita', e' restare in Olanda, senza la Raffa, la felicita'…e venire a trovarmi e mai piu' lasciarmi, la felicita', e andare poi insieme in Texas e a Londra, la felicita', felicita'!!! You know! Efcharistíes, for everything!

Beste Eric, Arian, Jo, Joost and Inge thank you very much for your support and interest in my project and the helpful discussion during our Monday morning work.

A special thanks to the lab-angels, who make everything working… Mariette (the God-mother of the lab), Truus (the most elegant lady in the lab), Ellen (the army officer of the lab), Riiiiiiiiiiiiiiinaaaaaaa (my favourite mosaic maker!!), Nicole (congratulations for the new addition in your family), Niels (also my neighbour in the Overvecht Royal Palace… come for an authentic Italian coffee before I leave), Joan (I'm sorry I've never been able to speak dutch with you…maar, veel geluk met

alles!!!) and Aukjie (the Mass-spec addict). And although she has retired, I would also like to thank my bench-mate Jet for entertaining us all with her singing and the nice time we spent talking about Italy.

Lieve Monique dG, Monique J, Linda and Ada, thanks a lot for all the "administrative" help you gave me and for helping me all the time I forgot something important (which happens very often!!).

Lieve Maui, my first student!! Thank you, you made me really proud! It has always been nice to talk about science...and to relax together with a cigarette! Good luck with your PhD!

Kooooeeeen...the first man of the FXR-obsessed group...good luck with everything!

Danielle, I wish you good luck with your new adventure in our wonderful FXR-geeks group!

The "Moschetta's family": Alessio, Annalisa, Andria, Antonella, Bellafantona, Chiara DG, Chiara sorellina b, Ciciona,Cissy, Fabiolina, Ilenia-Mamy (mpb), Lore(da)na (o Lorex!!), Marika, Mirk, Roberta, Salvigno, Simona, Stefanona, Stefanina (altra sorellina b), Vacca, zio Pinuzzo,...a tutti voi, grazie per avere reso i miei mesi ad SMI very enjoyable, nonostante i topi con la diarrea, i cateteri per il TNBS, il negozio di tabacchi che vende le scarpe e....tutto il resto! Mi mancate!

Beste allemaall van de maag darm en lever ziekten and pathologie afdelingen, Dr. Monkelbaan, Dr. Vleggaar, Niels, Maurice, Paul, Jantine, Jose', Martijn, Jan Steven, Pauline, Meike, Ellen, Sasha, Fiona, Mirthe, Lotte, Romy, Marco, Auke, Anke, Ofke, Marian, Claudia, Marguerite, Willem and Martin, thank you very much for your interest in my sometimes-too-basic projects! Our afdeling meetings have always been positively challenging for me…and I've learnt a lot from you!!!

Beste Rian, we shared a project during these years…and a chapter on our bokije!!! Good luck with everything and don't forget to invite me to your promotie!!!

Without my family in Hollandia, Eissa and Gianpi, my life in the last four years would have not been so enjoyable…shukkran…grazie! Eeeeiiiissssss…you are my best crazy friend. It has been a gift to meet you in Bilstraat, everything changed in my dutch life when I heard you laughing on the 3rd floor. Gianpi, marito mio! Non ci sono parole per dirti quanto ti adoro e quanto è stato bello condividere tutto con te, dalle lenticchie reidratate alle chiacchierate di ore sul divano, dalle bestemmie

in tutte le lingue del mondo, alla lavagna di chi sai tu con i grafici spazio-tempo-terza dimensione! It is comforting to know that either in the same place or not, our feeling for each other will never change. We'll always be a family! And don't forget, I love YOU more than Demi Moore!!!

Tina, my favorite m....tTIna! Thank you for all the crazy moments we spent together, from the butter cookies swimming in the nutella, to the million cigarettes we smoked together, to our Nederland-Deutschland chat! We are soooo lucky, let's dance!

Gabriele, il mio gastroenterologo milanese preferito! Da quando sei tornato in Italy il dipartmento non è stato più lo stesso! Spero di rivederti presto!

Lieve "Empty Moments" people, Dorothea, Paris, Yang, Ioana, Dilian, Wilco, Yuliya, Divya, Claire, thanks a lot for the wonderful time we spent together. I hope to share a stage with you again! I will never forget you!

Va & Ro...il nostro eterno sodalizio ha resistito anche alla mia fuga in Olanda! Com'e' bello sapere che nonostante la vita cambi e ci cambi continuamente ci sono cose che non verranno mai a mancare...come il nostro volerci bene! Grazie perche' ci siete sempre!

Papyno Stef, tu ci sei sempre stato...da vicino o a distanza...e sei stato fondamentale nel mostrarmi che c'è sempre un modo diverso, migliore, per interpretare la vita!

Reverendissima Madre benedetta Ileana, dopo la grande canzone "Mille pippe di te e di me", 3 sole parole che racchiudono un grande significato, di tutto quello che vorrei scriverti ma non posso (sai verba volant, scripta manent): ti voglio bene!

Francesca, grazie perche' tu sei una delle poche baresi che puo' veramente capire cosa ho in testa e perche'...e grazie anche alle tue folli paranoie!

Nonna....grazie perche' tu sapevi gia' da quando avevo cinque anni che sarei diventata una scienziata!!

Un grazie speciale ad una persona che ha sempre fatto il tifo per me, grazie ZioRI... questa tesi e' anche per te!!!

Mamma e Papà, con me avete preso il Diploma, la Laurea e ora anche il PhD! Come avrei fatto senza il vostro supporto? Grazie per aver creduto in me e nei mei sogni e per avermi lasciato libera di esplorarli. Grazie per aver ascoltato in silenzio tutte le

mie follie e per aver saputo scuotermi nei momenti più difficili. Voi siete i migliori!

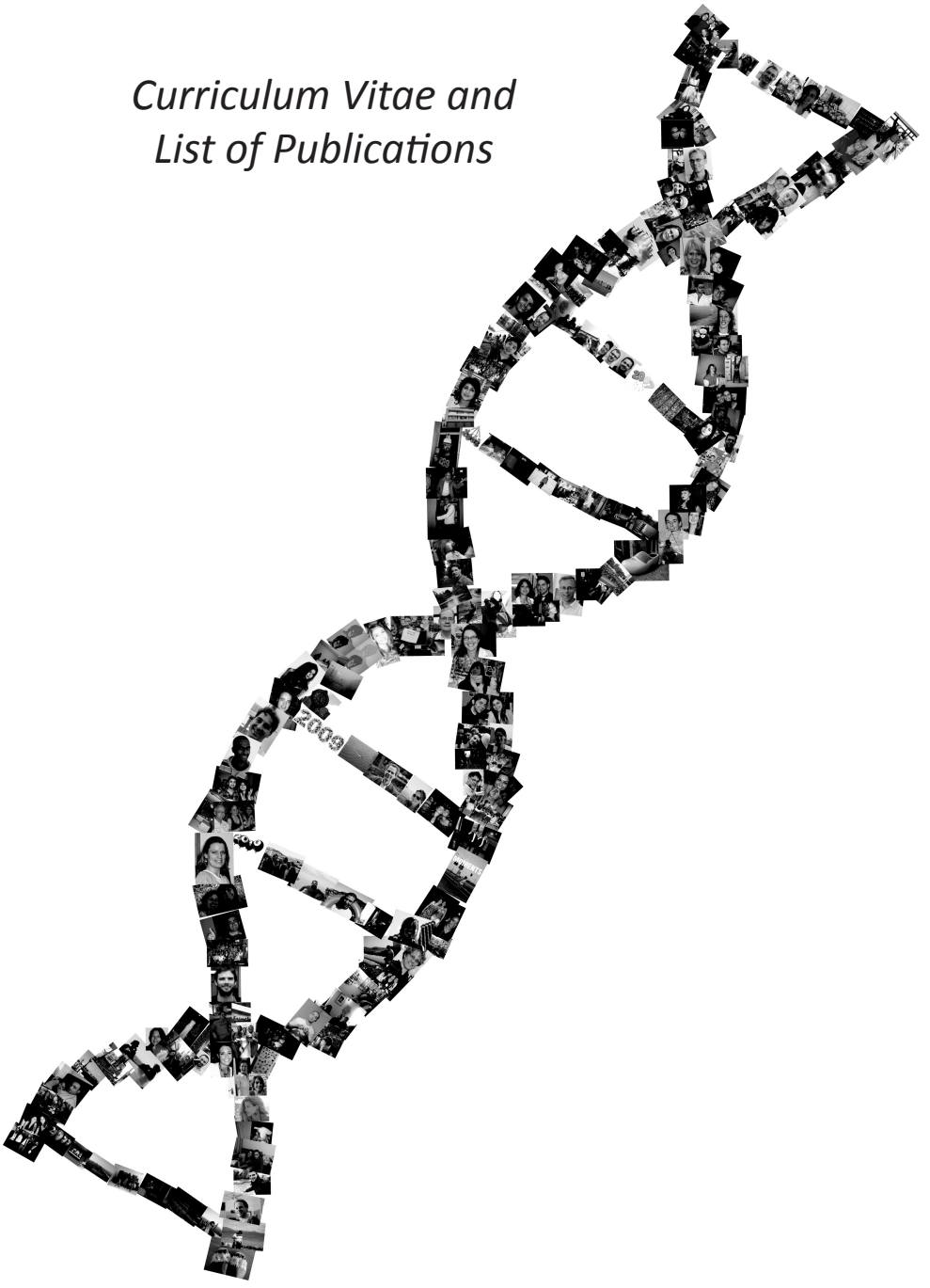
Daniele...tu sei TU. E questo e' di fondamentale importanza! Resta.

Vale, sorella, paraninfa, amore, tesoro! Tu sai. Tu sei la mia vita, altro io non ho! Ma noi due, amor mio, che siam poco, insieme siamo un po' di più...Tuuuu sei quellaaa, che si incontra una volta...Onlyyy youuu and you alone...se non avessi piu' te, meglio morire, questo devi sapere...da queste mie parole puoi capire quanto Ti amooo, ti amooooo!!!

And last, but not least, ma'a 7abibi 7azem. With you suddenly everything changed from the north-european grey, to colorful life. Together we shared our PhD lives… and much much much more. Laughs and tears, sun and rain…e poi ancora in alto con un grande salto! Life will bring us more…let's just see what's on the menu!!

Veel liefs,

Curriculum Vitae and
List of Publications



Raffaella Maria Gadaleta was born on February 2nd 1980, in Bari, Italy. She attended the Liceo Scientifico "Gaetano Salvemini" in Bari. After graduation, she studied "Biological Sciences" at the University of Bari. In 2005 she did an undergraduate scientific internship at the Department of Biochemistry and Molecular Biology "Ernesto Quagliariello" of the University of Bari. In July 2006, she graduated in Biological Sciences with a specialization in Physiopathology. After that she joined the Laboratory of Lipid Metabolism and Cancer, Department of Translational Pharmacology, at the Consorzio Mario Negri Sud, Santa Maria Imbaro (CH), Italy (Head A. Moschetta, MD, PhD). In March 2007 she started as a PhD student on a combined project between the Laboratory of Metabolic and Endocrine Diseases (Supervisor S.W.C. van Mil, PhD), the Department of Gastroenterology and Hepatology (Supervisor K.J. van Erpecum, MD, PhD) both at the University Medical Centre of Utrecht, The Netherlands, and the Laboratory of Lipid Metabolism and Cancer, at the Consorzio Mario Negri Sud (A. Moschetta, MD, PhD). The results of her research are described in this thesis.

Raffaella Maria Gadaleta e' nata a Bari (Italia) il 2 Febbraio 1980. Ha frequentato il Liceo Scientifico "Gaetano Salvemini" a Bari. Dopo il diploma, ha studiato "Scienze Biologiche" all'Universita' degli Studi di Bari. Nel 2005 ha iniziato l'internato per la preparazione della tesi di laurea al Dipartimento di Biochimica e Biologia Molecolare "Ernesto Quagliariello" all'Universita' di Bari. Nel Luglio del 2006 si e' laureata in Scienze Biologiche con una specializzazione in Fisiopatologia. Dopo la laurea si e' unita al gruppo di Antonio Moschetta (MD, PhD) a capo del Laboratorio di Metabolismo Lipidico e Tumori, facente parte del Departimento di Farmacologia Traslazionale del Consorzio Mario Negri Sud a Santa Maria Imbaro (CH), Italia. Nel Marzo del 2007 ha iniziato come studente PhD un progetto in collaborazione tra il Laboratorio di Malattie Metaboliche ed Endocrinologiche (Gruppo di Saskia van Mil, PhD), il Dipartimento di Gastroenterologia ed Epatologia (Gruppo di Karel van Erpecum, MD, PhD), entrambi facende parte del Centro Medico Universitario di Utrecht (Olanda), e il Laboratorio di Metabolismo Lipidico e Tumori al Consorzio Mario Negri Sud (Gruppo di A. Moschetta). I risultati delle sue ricerche sono descritti in questa tesi.

List of Publications

"Bile acid and their nuclear receptor FXR: relevance for hepatobiliary and gastrointestinal diseases"
Gadaleta RM, van Mil SWC, Oldenburg B, Siersema PD, Klomp LWJ, van Erpecum KJ
Biochim Biophys Acta, Molecular and Cell Biology of Lipids, 2010 Jul

"Farnesoid X Receptor activation inhibits inflammation and preserves the intestinal barrier integrity in inflammatory bowel diseases"
Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen ECL, Renooij W, Murzilli S, Klomp LWJ, Siersema PD, Schipper MEI, Danese S, Penna G, Laverny G, Adorini L, Moschetta A, van Mil SWC
Gut, 2011 Apr

"Deciphering the nuclear bile acid receptor FXR paradigm"
Modica S, **Gadaleta RM**, Moschetta A
Nuclear Receptors Signal, 2010 Nov

"Activation of bile salt nuclear receptor FXR is repressed by inflammatory cytokines activating NF-κB signaling in the intestine"
Gadaleta RM, Oldenburg B, Willemsen ECL, Spit M, Murzilli S, Salvatore L, Klomp LWJ, Siersema PD, van Erpecum KJ[#], van Mil SWC[#]
Biochim Biophys Acta, Molecular Basis of Diseases, in press

"FXR activation and FXR genetic variation in inflammatory bowel disease"
Gadaleta RM[*], Nijmeijer RM[*], van Mil SWC, van Bodegraven AA, Crusius JBA, Dijkstra G, Hommes DW, de Jong DJ, Stokkers P, Verspaget HW, Weersma RK, van der Woude CJ, Stapelbroek JM, Schipper MEI, Wijmenga C, van Erpecum KJ, Oldenburg B
Submitted to 'Inflammatory Bowel Disease'

"Expression of the bile salt nuclear receptor FXR in the intestine of patients with primary sclerosing cholangitis and coexistent inflammatory bowel disease"
Gadaleta RM, Oldenburg B, Renooij W, Schipper MEI, Willemsen ECL, Klomp LWJ, Siersema PD, van Mil SWC, van Erpecum KJ
Submitted to 'Liver International'

"Phosphorylation of FXR-S224 is necessary for transactivation of bile salt homeostasis gene but not for inhibition of NF-κB-mediated inflammatory signalling"
Gadaleta RM, Willemsen ECL, van Gent R, Spit M, Brenkman AB, van Mil SWC
Manuscript in preparation

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